HYALOFAST™ , a new biological appoach for chondral and osteochondral lesion treatment

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ABSTRACT

The field of conservative treatments for osteochondral and chondral lesion presents different approaches, many of which involve the contribution of cells directly at the lesion site. Specifically, autologous stem cells represent an attractive cell source due to their potential to differentiate both towards bone and cartilage and the possibility of representing a single step treatment.

Hyalofast™, a HYAFF11® based scaffold, is a support for autologous mesenchymal stem cells (MSCs) in the treatment of osteochondral and chondral lesions.

Preclinical *in vitro* and *in vivo* data have been collected to characterize Hyalofast™ and demonstrate that, thanks to HYAFF11® biological properties and structural role, MSCs can properly regenerate chondral as well as osteochondral lesions, via successful differentiation towards the involved tissue lineages. Prospective clinical experience in the use of Hyalofast™ for the one- step treatment of osteochondral lesions of the ankle and of the knee have been reported and confirmed the safety of the treatment and its short term good results in terms of clinical outcome and MRI assessed defect healing.

In conclusion, based on preclinical and clinical evidences, Hyalofast™ appears a promising biological prostheses to be used in combination with autologous MSCs for the treatment of chondral and osteochondral lesion. Long term clinical data will be necessary to prove result durability.

Introduction

Chondral and osteochondral defects are joint lesions that can cause pain and functional disability, with the potential progression to an early osteoarthritis (OA). Diseases of the articular joints present a major medical, social and economic burden of the society.

Partial-thickness articular cartilage defects do not heal spontaneously and degenerate over time. Small chondral lesions that involve the subchondral bone may fill up with fibrocartilage and

render to patient as asymptomatic, but the large osteochondral defects are less likely to benefit from fibrocartilaginous healing response.

Different approaches have been proposed to treat osteochondral lesions such as debridement, drilling, chondroabrasion, microfracture and curettage, but none of those has been able to restore the physiological osteochondral tissue. Basically, hyaline cartilage repair has been achieved through cartilage replacement (which is a type of mosaicplasty) (1) or cartilage regeneration through autologous chondrocyte implantation (ACI) (2,3). Those techniques are valuable for large cartilage lesions but, as they are based on chondrocytes, need to be associated to a parallel treatment for the subchondral bone layer portion of the defect.

Recently, bone-marrow-derived mesenchymal stem cells have been identified as a new option for the treatment of osteochondral defects, thanks to their ability to differentiate towards bone and cartilage and, therefore, to repair at the same time both the chondral and bone layer of the lesion (4,5). The use of MSCs is clinically appealing as the possibility of concentrating bone marrow aspirate directly during the surgical procedure or to recruit MSCs at the defect site via microfracture of perforation are one-step procedures, which avoid the need of two operations and contain surgery related costs. For the success of both the approaches, it is necessary to guarantee that bone-marrow MSCs are maintained *in situ* after direct mobilization or application of the bone marrow aspirate, avoiding cell dispersion in the articular space. Hyalofast™ , a non-woven scaffold based on HYAFF11®, the benzylic ester of hyaluronic acid, has been developed for this purpose and to support cell engraftment and differentiation.

Hyaluronic acid (HA) is a major component in chondrocytes extracellular matrix (ECM) organization and it is a chondroinductive factor responsible for modulating pericellular matrix before cell condensation. HYAFF11® is a biomaterial with excellent biocompatibility and biodegradation properties that allow its resorption at the lesion site through physiological metabolic processes (6). Hyalofast™ has structural and chemical properties suitable for the treatment of the lesions of the osteochondral compartment. In fact, in parallel to its bioresorption, the remodeling of the chondral and osteochondral tissue is promoted, thanks to the peculiar informative properties of the HYAFF11® scaffold.

Hyalofast™ is a user-friendly matrix that can be applied with mini-invasive procedures, such as mini-arthrotomy or arthroscopy, acting as a biological biodegradable prosthesis which guides tissue remodeling and the healing of both the bone and cartilage tissue through the action of the MSCs, which have populated the scaffold.

In this review, published preclinical characterization data and clinical experiences referred to the use of Hyalofast™ in the treatment of osteochondral lesions of the ankle and of the knee will be presented and discussed.

Preclinical data

In vitro characterization

Preliminary *in vitro* studies have been performed to evaluate the suitability of Hyalofast™ as support for stem cell engraftment and its role in differentiation towards the chondral and bone phenotypes, fundamental requirements for Hyalofast™ clinical use in combination with one-step orthopedic procedures.

Rat MSCs have been seeded on Hyalofast™ and their attachment and viability on the scaffold evaluated during *in vitro* culture (7). Cell attachment and growth on the HYAFF-based support were analyzed showing that Hyalofast™ support adhesion, migration and proliferation of MSCs, as well as the synthesis and delivery of extracellular matrix components.

In other experiments (8,9,10), chondrogenic differentiation of human MSCs was induced using TGFb1, whose chondroinductive effects have been well established in MSCs, and the process was analyzed at different time points (24 hours and 7, 14, 21 and 28 days) using microscopy techniques, real-time PCR and immunohistochemistry. Results demonstrate that MSCs chondrogenesis on Hyalofast™ is well induced by TGFb1, favoring the *in vitro* formation of a cartilage like construct. Hyalofast™ favors the chondrogenic differentiation of MSCs, allowing them to express typical chondrogenic markers of such as collagen type II, IX, aggrecan and Sox-9 mRNAs. At the same time the expression of collagen type I is down-regulated, as observed in physiological hyaline cartilage. It has to be noted that protein expression of collagen type II is observed already on day 14 in a few areas and on day 28 in the entire scaffold, suggesting that MSC differentiation is very fast.

Regarding the differentiation towards the osteogenic lineage, rat bone marrow stromal cells (BMSCs) have been cultured on Hyalofast™ in the presence of bFGF and *in vitro* mineralization assessed (11). Specifically, osteoblastic differentiation was evaluated by electron microscopy and immunohistochemical analysis and by measuring cellular proliferation and alkaline phosphatase activity and mRNA expression of osteogenic markers. Results indicate that culture of rat progenitors cells on Hyalofast™ in the presence of bFGF leads to the differentiation of BMSCs into osteogenic cells producing a mineralized matrix after a single month in culture. This suggests that Hyalofast™ is a suitable support to maintain *in situ* progenitor cells that can differentiate into osteogenic cells in the presence of appropriate stimulation.

Taken together, the above data show that, in *in vitro* culture in the presence of suitable stimulation, MSCs seeded on Hyalofast™ are able to attach, grow and differentiate towards both the chondrogenic and osteogenic lineages. The differentiation takes place in a fast way, as demonstrated by the early expression of typical markers. These findings demonstrate that Hyalofast™ can be adequate for the regeneration of osteochondral lesions were both the cartilage and bone layer are affected.

The interaction between human mesenchymal stem cells and Hyalofast™ has been analyzed *in vitro* demonstrating a specific informative role of Hyalofast™ in the modulation of inflammatory and degenerative factors (12). A direct action of Hyalofast™ has been demonstrated on hMSCs in modulating the expression and release of both CXC chemokines/receptors such as CXCL12 (SDF1)/CXCR4, CXCL13/CXCR5 and catabolic/inhibiting factors as MMP3, MMP13 and TIMP1. These data suggest that the use of Hyalofast™ in tissue repair might favor the engraftment of specific precursor cells in the damaged area by the regulation of biological signaling.

Recently, a study compared articular chondrocytes, MSCs from human bone marrow (BM) and adipose tissue (AT) seeded on a HYAFF11® scaffold for their ability to express genes and synthesize proteins associated with chondrogenesis (13). Cells were expanded in monolayer cultures and after seeding on the scaffold the chondrocytes were maintained in plain medium, while the two MSC populations were given a chondrogenic differentiation medium. Chondrogenesis was assessed by real-time RT-PCR for chondrocyte-associated genes, by immunohistochemistry and by ELISA for collagens in the supernatant. Data show that, when cultured in chondrogenic differentiation medium in a HYAFF11® scaffold, MSCs respond with up-regulation of chondrogenic markers measured at the mRNA level. For maximal effect, MSCs need to be continuously exposed to the chondrogenic differentiation medium. In the conditions of the study, chondrogenesis in HYAFF11® scaffolds was more efficient using BMSCs than AT-MSCs or chondrocytes, nevertheless it has to be considered that in this study chondrocytes were culture in different conditions, with a plain culture medium. In conclusion, it is possible to confirm that BMSCs seeded on Hyalofast™ can optimally differentiate towards the chondrogenic lineage in the presence of suitable stimulation.

***In vivo* characterization**

In order to confirm that the *in vitro* observations are actually reflecting the *in vivo* properties of the scaffold, animal studies have been performed to assess the role of Hyalofast™ in the regeneration of osteochondral lesions.

It is hypothesized that HA-based polymers recreate an embryionic-like milieu allowing host progenitors cells to regenerate the damaged articular surface and underlying bone. To prove this concept, in 2000 the group of Solchaga performed an *in vivo* study (14) specifically focused on osteochondral defects created on the femoral condyles of 4-month-old rabbits. Partial thickness defects do not penetrate the subchondral plate and do not have access to reparative cells, while full-thickness osteochondral lesions like the ones created in this animal model have access to reparative cells of the marrow. This allows the mobilization and recruitment of progenitors cells that can proliferate and differentiate within the injured site. In this study, HYAFF11® scaffolds were placed to fill the defects and results were evaluated at 4 and 12 weeks after surgery, untreated controls were used for comparison. Four weeks after implantation the defects treated with HYAFF11® scaffold presented a top layer composed of either hyaline cartilage or fibrocartilage, that evolved in hyaline-like cartilage at 12 weeks after surgery, whereas the untreated defects presented fibrous tissue or fibrocartilage both at 4 and 12 weeks. Histological scores were significantly higher in defects treated with HYAFF11® than in the untreated lesions. The introduction in the osteochondral defects of the HYAFF11® matrix improves the regenerative process by providing a scaffold with appropriate physical structure and chemical composition for cell organization in a mechanically demanding environment and stimulation for their differentiation. Moreover, it is possible that hyaluronic acid oligomers derived from HYAFF11® degradation might influence in different ways cartilage tissue formation. Firstly, the oligomers might affect angiogenesis and bring blood borne cells to the defect's edge where there is the local release of proteases which facilitate molecular integration. Secondly, the oligomers could act as chemoattractant for a special class of mesenchymal progenitor cells, supposedly from the synovium or the edge of the condyle, that are capable of forming articular cartilage.

In another study, osteochondral defects were created in the medial femoral condyles of young adult rabbits and lesions were treated with HYAFF11® scaffolds loaded with autologous bone marrow derived culture expanded mesenchymal progenitor cells (15). In comparison with defects treated with the unloaded HYAFF11® scaffold, defects treated with cell-loaded scaffolds appear to develop chondrogenic tissue more rapidly and the integration with host bone and cartilage is superior. In general, despite the model lesion allows for MSCs homing from the subchondral bone into the scaffold at the defect site, cell-seeded HYAFF11® scaffolds originate more uniform new tissue and in a shorter time. This suggests that the seeding of MSCs on the HYAFF11® scaffold accelerates the regenerative response, avoiding the delay caused by the time required for precursor cells to migrate and populate the support.

On the contrary, another paper reported the experience of a study where osteochondral defects in the rabbit were treated with Hyalofast™ alone or Hyalofast™ seeded with MSCs (16) and results in terms of filling degree do not show any difference. In this animal model, cells from the subchondral bone marrow migrated to the lesion site and may have contributed to the repair in such an important way that partly outweighed the effect of the added cells. It has to be noted that the animals of the previously mentioned study were much younger at the time of defect creation (4 month old versus 24 month old animals of the other study), therefore it is reasonable to hypothesize that their BM is richer of MSCs, leading to a predominant role of the loaded scaffolds respect to the latter experiment where animals were adult and could have benefit of a less relevant BMSCs contribution.

Recently, an *in vivo* experiment in a rabbit model showed that MSCs seeded on Hyalofast™ can also positively interfere with osteoarthritis progression (17). The surgical osteoarthritis model was based on anterior cruciate ligament transaction (ACLT). Upon surgery animals were divided into 4 groups: sham operated, ACLT, ACLT – Hyalofast™ only and ACLT- Hyalofast™ with MSCs. It has to be noted that, in this model, in the scaffold only group there is no access to the subchondral bone, therefore no MSC population could come in contact with the lesion area. After 3 and 6 months, histomorphometrical and immunohistological results show statistically significant influence of the MSC-loaded Hyalofast™ on disease progression by the formation of cartilaginous tissue at the site of the lesion.

Published *in vivo* experience with HYAFF11® scaffolds suggests that the interaction of the MSCs with the biomaterial promotes their engraftment and induces their chondrogenic differentiation, while the mechanical activity and synovial milieu provide the environment that controls the regeneration process kinetics. It is also proposed that MSCs progress through the differentiation cascade to hypertrophic chondrocytes, which are replaced by vasculature and host progenitor cells that fabricate bone at the bottom of the defect, while the microenvironment at the surface provides the signals that inhibit the final progression of the MSCs, thus maximizing the articular chondrocytes phenotype.

Clinical data

A new one-step arthroscopic technique for the treatment of chondral and osteochondral lesions in the ankle and in the knee has been developed using Hyalofast™ as support for concentrated bone marrow derived cells. Autologous bone marrow has been selected as MSC source as it provides not only stem cells but also accessory cells that support angiogenesis by the release of growth factors. The procedure involves harvesting of bone marrow from the patient's iliac crest and its centrifugation during the surgical session to obtain a concentrated aspirate, enriched in

bone marrow derived cells, to be loaded onto Hyalofast™ and then applied to fill the osteochondral defect. The technique does not involve the use of suture, while autologous platelet-rich plasma (PRP) gel can be used to provide useful growth factors for progenitors cell differentiation and help fixation of Hyalofast™ in the lesion site, if necessary.

Preliminary prospective clinical data on talar osteochondral lesions are available on 25 patients (18, 20). The authors performed a lab characterization in order to verify *in vitro* the capability of the biomaterial to support BM- derived cells. Lesion area and depth, previous surgery and the American Orthopaedic Foot and Ankle Society (AOFAS) score were reported at baseline, while the ability of regenerate osteochondral tissue was evaluated during follow up with clinical evaluation and using MRI. Despite there is no control group, it is generally agreed that, for the type of lesion included (Type II), surgical treatment is mandatory. After 2 years follow up, the authors suggest that the patients treated with the implantation of the BM derived cells on Hyalofast™ resulted in statistically improved AOFAS score similar to those reported for other widely used techniques (i.e. microfracture, chondroplasty, etc) at the same early time point. The patients have been followed up to 36 months and clinical observations demonstrate that AOFAS score not only improved significantly after the procedure (18), but after 36 months improvement increased further (20). MRI results after 2 years from treatment confirm the complete filling of the defect and the regeneration of both the cartilage and subchondral bone layer (18). MRI observations after 36 months confirm that there has not been an arthritis progress, while 76% of the cases show an almost complete integration of the regenerated tissue with the host cartilage (20).

An additional study aimed at characterizing the reparative tissue obtained with the MRI T2-mapping sequence and verifying a possible correlation of those with clinical results (19). MRI T2 mapping technique provides additional information respect to the standard MRI on the properties of the regenerated or repaired tissue. This technique can identify differentially the percentage of regenerated tissue similar to physiological hyaline cartilage, the percentage of remodeling/inflammatory tissue, the percentage of fibro-cartilaginous tissue and the percentage of newly-formed bone. For the purpose of assessing results in parallel to normal cartilage, 20 healthy volunteers were recruited and compared to 20 patients which received Hyalofast ™ in the Giannini‘s study (20). After 2 years from the treatment, the analysis via T2 mapping indicates that the regenerated tissue has characteristics comparable to the healthy hyaline cartilage. T2 mapping results were then correlated with AOFAS clinical score results and a good match is observed.

In summary, available clinical data are promising in view of a potential long term value of the treatment of osteochondral defect in the ankle with Hyalofast ™ loaded with autologous bone marrow aspirate. To confirm durability of the treatment, a longer follow up is needed, as indicated by recent clinical evidences suggesting that 3 years can be considered a threshold between short term results achievable with simple techniques, such as microfractures (21), and long term stable results typical of regenerative tissue engineering approaches, such as ACI (22).

Following the same protocol applied in the ankle, Hyalofast™ has been used in the treatment of osteochondral defect in the knee (23). Twenty consecutive patients who had grade-III or grade-IV osteochondral lesions according to the ICRS classification system have been treated with the one step surgical technique involving the application of Hyalofast™ loaded with autologous bone marrow aspirate. Eighteen patients presented post-traumatic lesions, while two patients were affected by osteochondritis dissecans. Assessment consisting in clinical evaluation with the IKDC subjective questionnaire and the KOOS questionnaire was performed before surgery and at 6, 12, 18 and 24 months postoperatively. A magnetic resonance imaging scan was acquired for all patients in the study preoperatively, at 6 months, at 12 months and at the time of final follow-up. Imaging sequences were carried out according to the MOCART scoring system. Additionally, the first two consecutive patients underwent a second-look arthroscopy and a biopsy 12 months after surgery. A significant improvement is found in both the IKDC score and the KOOS score from the time of testing before the operation to the time of testing at each of the follow-up visits ($p < 0.0005$). Magnetic resonance imaging examination shows satisfactory growth of bone and cartilage, nearly complete defect filling and satisfactory integration of the graft in 80% of patients at the time of follow-up. Additionally, both the histochemical as well as the immunohistochemical biopsy specimens show regenerated cartilage tissue in an advanced remodeling phase. Immunohistochemical analysis confirms the presence of proteoglycans and type-II collagen, which are well-recognized markers of healthy hyaline cartilage.

Although further studies with longer follow-up are required, available data demonstrate that the application of Hyalofast™ loaded with concentrated autologous bone marrow aspirate is a good and reliable option for the treatment of osteochondral lesions of the knee.

Discussion and conclusions

Osteochondral and chondral lesions represent a serious concern as they affect relatively young people and, if not correctly treated, can lead to permanent disability with the development of osteoarthritis affecting seriously quality of life and accounting for a relevant health cost. According to lesion dimension and history (i.e. first line versus second line), orthopedic surgeons are using dedicated therapeutic approaches which are aimed to prevent damage degeneration and

promote tissue regeneration. In the case of large lesions, such as with area over 4 cm^2, and/or demanding defects that failed previous treatments or affect athletes and very young people, the use of cellular therapies, such as ACI, is supported by an extensive clinical experience and long term follow up data (22, 24). In the other cases, orthopedics are now endorsing more and more the use of one step procedures which allow to save time and reduce costs, performing in a single surgical session all the actions which are needed to treat the patient.

Very small first line lesions, with area less than 2 cm^2, are generally treated with microfracture in order to create patent passages to allow precursors cells from the subchondral bone to move towards the defect area and play their role in tissue healing. The amount of cells which can be recruited with this strategy is limited and probably they are not rebuilding the tissue structure themselves, but they possibly act in a paracrine way to produce regenerative stimuli on other resident cells. One of the technical difficulties with microfractures is to keep them in place while protecting the tissue which is regenerating. In the case this procedure is applied, the surgeon should be sure that the cells are actually homing in the lesion site and avoid their dispersion in the joint space. These important features can be assured by the application of a chondroprotective coverage onto the area treated with microfractures. The rationale is that the cover can trap MSCs coming out from underneath drillings, keeping them in place and behaving as a scaffold while they differentiate into chondrocytes, which will regenerated a healthy cartilage. Hyalofast™ can be applied over microfracture to maintain *in situ* MSCs after their mobilization, as supported by the successful application of HYAFF11® scaffolds in osteochondral defect with access to subchondral bone MSCs (15). Recently the use of other biocompatible stabilizing covers, analogue to Hyalofast™ and made of hyaluronan and other materials (i.e. collagen), was reported in large animals and then introduced in the clinical practice (25). Correspondent results confirm that the acellular cover can be a promising treatment option for cartilage defects improving the regeneration of articular cartilage.

For larger defect, with area ranging from 2 up to 4 cm^2, orthopaedic surgeons usually prefer a stronger approach that could potentially involve a large number of precursors cells, ideally enriched with other biological molecules, to support a faster recovery of the damaged area. This could be achieved by the application of concentrated bone marrow aspirate that contains MSCs, able to differentiate towards chondrogenic as well as osteogenic lineages, along with a concentrated cocktail of growth factors. As documented in the recent clinical experience, the use of Hyalofast™ as support for bone marrow aspirate, not only provides a useful tool for an easy handling and application of the bone marrow, but also favours cell engraftment and organization.

In conclusion, Hyalofast™ is a medical device used in the reconstruction of cartilaginous defects which may involve the underlying bone tissue. Thanks to its structure and composition, Hyalofast™ acts as a chondral bioresorbable substitute and a matrix which promotes osteoblastic differentiation in the deeper bone layer, obtaining the reconstitution of a biofunctional tissue.

Hyalofast™ is capable of acting as a scaffold for the *in situ* attachment and differentiation of the undifferentiated mesenchymal cells coming from the subchondral compartment after their mobilization due to microfracture and/or perforation procedures or of autologous bone marrow aspirate, according to the concept of the guided regeneration of the tissues. The use of Hyalofast™ in combination with MSCs allows to treat joint defects with a new one-step approach, avoiding the cost and complexity related to two-step techniques.

Preclinical and clinical data support the safety and short term efficacy of using Hyalofast™ in the treatment of chondral and osteochondral lesion and long term follow up will be useful to assess the result durability.

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