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An amidated carboxymethylcellulose hydrogel for cartilage regeneration

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Abstract An amidic derivative of carboxymethylcellulose was synthesized (CMCA). The new polysaccharide was obtained by converting a large percentage of carboxylic groups (~50%) of carboxymethylcellulose into amidic groups rendering the macromolecule quite similar to hyaluronan. Then, the polysaccharide (CMCA) was crosslinked. The behavior of CMCA hydrogel towards normal human articular chondrocytes (NHAC) was in vitro studied monitoring the cell proliferation and synthesis of extra cellular matrix (ECM) components and compared with a hyaluronan based hydrogel (Hyal). An extracellular matrix rich in cartilage-specific collagen and proteoglycans was secreted in the presence of hydrogels. The injectability of the new hydrogels was also analysed. An experimental in vivo model was realized to study the effect of CMCA and Hyal hydrogels in the treatment of surgically created partial thickness chondral defects in the rabbit knee. The preliminary results pointed out that CMCA hydrogel could be considered as a potential compound for cartilage regeneration.

1 Introduction

Articular cartilage is the thin layer of deformable, load bearing material, which lines the bony ends of all diarthrodial joints [1]. It is well-known that in pathological processes, such as osteoarthritis (OA), the molecular weight and concentration of hyaluronan in the synovial fluid can be reduced due to the accumulation of liquid derived from the inflamed synovial vessels in the joint cavity. The result is a reduction in the viscoelasticity of the fluid and an increased susceptibility of cartilage to breakdown [2]. In order to counteract this process, a therapeutic strategy was started in the late 1960's, based on the use of intra-articular injections of exogenous hyaluronan (Hyal) [3]. Since then, several commercial preparations of Hyal, which differ in the polysaccharide molecular weight, have been developed. This therapy has been claimed to be effective in pain relief and in improving joint-motion [4–6]; on the other hand, several intra-articular administrations of Hyal are usually required in patients because of the rapid clearance of the degradable polysaccharide [7, 8]. An improvement was obtained by cross-linking the Hyal. This process guaranteed a longer effect of the polysaccharide in the action site and consequently, a reduced risk of infection due to numerous injections [9, 10]. Nevertheless, the cross-linking process slows down the hyaluronidases attack on Hyal chains, but does not prevent it. Consequently, an ideal substitute should present physicochemical characteristics similar to Hyal ones, but without its degradability. Moreover, as in the case of Hyal, the capability of stimulating chondrocyte proliferation and extracellular matrix (ECM) synthesis is to be highly wondered.

A previous study evidenced that the amidic derivative of the carboxymethylcellulose (CMCA) in the form of hydrogel showed a physicochemical behavior very similar to that of Hyal based hydrogel, but without undergoing such a rapid degradation [11]. The hydrogel behavior towards normal human articular chondrocytes (NHAC) was analyzed by monitoring the cell proliferation and synthesis of ECM components. In fact, articular chondrocytes orchestrate a

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balance between ECM synthesis and breakdown that facilitates the normal tissue metabolism [12]. Chondrocytes secrete both the ECM components (mainly type II collagen and proteoglycans) and the enzymes that can degrade the matrix. Therefore, they are responsible for the normal homeostasis of the tissue, and the intimate relationship among ECM proteins is critical to the function of articular cartilage [12]. The latter undergoes degradation in response to numerous stimuli, including trauma and degeneration. When the balance between the anabolic and catabolic activity is in favor of tissue catabolism, ECM becomes compromised and is less able to withstand mechanical stress, by that resulting in an accelerated degradation. Local growth factors are integral to normal cartilage metabolism, while in degeneration, there is an increase in inflammatory cytokines that inhibit collagen and proteoglycan synthesis. Cytokines as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) also stimulate the expression of matrix metalloproteinases (MMPs) that directly degrade cartilage and also stimulate other enzymes such as cycloxygenase-2 and nitric oxide synthetase, which in turn cause further tissue catabolism and damage [12]. An experimental in vivo model was realized to study the effect of CMCA hydrogel in the treatment of surgically created partial thickness chondral defects in the rabbit knee. These defects resemble the clefts and fissures observed during the initial stages of OA and do not heal spontaneously [13].

2 Materials and methods

2.1 Materials

The sodium salt of CMC (viscosity 402 mPa.s in 2% w/v aqueous solution at 25°C and carboxymethylation degree of 0.9 ± 0.1 per monosaccharide unit, Mw = 200000 D) was supplied by Hercules Italia S.p.A (Italy). The sodium hyaluronate (Hyal-Na, MW $\approx$ 180000 D) was provided by Biophil S.p.A. (Milan, Italy). All the other reagents were purchased from Fluka Chemie AG (Switzerland).

2.2 Methods

2.2.1 Realization of the amidic carboxymethylcellulose (CMCA) based hydrogel

2.2.1.1 Amidation of polysaccharide The procedure used to obtain the amidic derivative of CMC was previously reported [11]. Briefly, the CMC tetrabutylammonium salt (CMCTBA) was dissolved in *N,N′*-dimethylformamide (DMF) under mechanical stirring and a nitrogen flow. Once the polymer was completely dissolved, the temperature was lowered to 4°C. The activating agent, 2-chloro-N-methyl pyridine iodide (CMP-J), was added in a stoichiometric amount compared to the percentage of carboxylic groups to be converted in amidic moieties. 1:1 equivalents of the amidating agent, alcoholic methylamine solution, were added together with two to three drops of catalyst (triethylamine). The reaction was maintained for 3 h and then the polysaccharide (CMCA) was precipitated with 95% ethanol overnight at 4°C. The precipitate was separated by centrifugation (1,500 rpm for 15 min), dissolved in water and dialyzed against NaCl 0,1M, for 48 h.

2.2.1.2 Cross-linking of CMCA and Hyal The cross-linking reaction was carried out dissolving the polysaccharide in the acidic form (CMCAH or HyalH) in a mixture DMSO:isopropanol (1:3) under mechanical stirring and a nitrogen flow. Then, the same activating agent, 2-chloro-N-methyl pyridine iodide (CMP-J), used in the amidation phase, was added in order to activate the free carboxylic groups. The cross-linking agent, 1.3 diamino propane, was added in a stoichiometric amount with the free carboxylic groups which had to be crosslinked. Two to three drops of catalyst (triethylamine) were added and the reaction was maintained for 3 h. The obtained hydrogels were purified by following washings with bidistilled water and ethanol.

2.2.1.3 Thixotropic behavior To evaluate the thixotropic behavior of CMCA and Hyal based hydrogels a loop test was performed on the samples, in the swollen state (PBS pH 7.4), using a strain-controlled AR2000 Rheometer (TA-Instruments, Leatherhead, United Kingdom) in the parallel plate configuration. Smooth and rigid plates were used for testing. The plates were impermeable to fluid flow so to reduce the free surface of the sample and minimize dehydration during testing. The G′ (storage modulus) and G″ (loss modulus) were recorded by increasing linearly the applied stress from a minimum (0,636Pa) to a maximum (10000Pa) value. Then, the applied stress was decreased, at the same rate, to the initial value [14].

2.2.1.4 In vitro study on NHAC Freeze dried samples of CMCA and Hyal [10] hydrogels were sterilized in ethanol 70% (4°C, 1 h), and air dried under a laminar flow hood. Then, all the samples were positioned to cover the bottom of 6 wells per each biomaterial (20 mg/well) in the 24-well-plates, and were completely hydrated to reconstitute hydrogels with cell culture medium (1 ml/well) used for the experiment 24 h before seeding cells.

A normal human primary chondrocyte culture (NHAC-kn, Clonetics™ Cell System, BioWhittaker Italia, Caravaggio, BG, I), derived from the human knee articular cartilage was used for the experiment.

Cells were expanded in monolayer cultures, using the Chondrocyte Growth Medium (CGM, containing FBS 5%,

gentamicin sulfate-amphotericin B 0.1%, bFGF-B 0.5%, R^3-IGF-1 0.2%, insulin 0.2%, transferrin 0.1%). When they reached 80% confluence, cells were trypsinized and counted (Coulter Counter Z1, Beckman Coulter Inc., Miami, FL, US). A cell suspension of 2×10^4 cells/ml at the first passage was seeded in each coated well and cells were mixed through the hydrogel. The same concentration of chondrocytes was seeded in empty wells as control (CTR). Chondrocyte Differentiation Medium (CDM, containing FBS 5%, gentamicin sulfate-amphotericin B 0.1%, TGFβ-1 0.5%, R^3-IGF-1 0.2%, insulin 0.2%, transferrin 0.2% and ascorbic acid 2.5%) was added and cultures were incubated 14 days in standard conditions (37°C in a humidified 95% air/5% CO_2 atmosphere), with the medium replaced every 3 days.

Supernatant from six wells per each hydrogel was collected after 7 and 14 days for the following tests: Collagen type II C-propeptide (CPII Elisa, IBEX Technologies Inc., Montreal, Quebec, CDN), Aggrecan (PG, Elisa Immunoenzymetric assay, Biosource, Nivelles, B), Interleukin 1β (IL-1β, Quantikine IL-1 Immunoassay, R&D Systems, MN, US), pro-Matrix Metalloproteinase-1 (MMP-1 Immunoassay, R&D Systems, MN, US), pro-Matrix Metalloproteinase-13 (MMP-13 Immunoassay, R&D Systems, MN,US), Cathepsin B, Immunoassay, R&D Systems, MN, US). Measured concentrations were normalized by cell proliferation value for each group [15].

The Cell Proliferation reagent WST-1 test was performed to assess cell proliferation and viability: l00 µl of WST-1 solution and 900 µl of medium (1:10 final dilution) were added to cultures, and the samples incubated at 37°C for another 4 h. Supernatants were quantified spectrophotometrically at 450 nm with reference wavelength at 640 nm. Results were reported as optical density (OD).

2.2.1.5 Experimental in vivo study (a) The study was carried out in compliance with the European and Italian Laws on animal experimentation and the principles stated in the Guide for the Care and Use of Laboratory Animals. Sixteen eight-month-old New Zealand adult-male rabbits (4.1 ± 0.5 kg body weight) were used. They were housed individually in stainless-steel cages and maintained under the same environmental conditions (temperature 20 ± 0.5°C, relative humidity 55 ± 5%). The animals were fed with a standard rabbit diet and filtered water ad libitum.

All surgical procedures were performed in aseptic conditions. The general anaesthesia was induced by an intramuscular injection of 44 mg/kg b.w. ketamine (Ketavet, Farmaceutici Gellini, Aprilia, LT, Italy) and 3 mg/kg b.w. xylazine (Rompun, Bayer Italia, SpA, Milan, Italy), maintained by means of a mixture of 2% isoflurane (Forane, Abbott Italia SpA, Campoverde LT, Italy) and oxygen/nitrous oxide (l/0.4L/min), and delivered by an automatic ventilator using a specially designed mask. The right femoral medial condyle of all rabbits was exposed by lateral-longitudinal skin incision. Using an operative microscope, the weight-bearing surface of each medial femoral condyle was lightly scarified with a sharp scalpel blade to obtain a chondral defect of 5×4 mm, without damaging the subchondral bone. Then, the surgical wounds were sutured. Immediately following surgery, eight animals (CMCA group) received CMCA hydrogel (20 mg freeze dried CMCA 1.2 mg /0.6 ml NaCl 0.9%), by percutaneous injection in the synovial capsula. The other eight were treated under the same conditions using NaCl 0.9% as control (Control Group). The treatments were repeated every 10 days, for three (animals sacrificed after 30 days) and five times (animals sacrificed at 50 days). The rabbits were allowed to move freely in their cages and all of them received antibiotics (Flumeqione-Flumexil, ATI s.r.l., Ozzano, BO, Italy) and analgesics (Orudis-Ketoprofene, Rhone Poulenc-Rorer, Milan, Italy) for two days. During the post-operative period, animals were checked on a daily basis by a veterinarian. At the end of the experimental times (30 and 50 days), four animals per group were euthanized (Tanax, Hoechst, Frankfurt a M., Germany) after induction of general anaesthesia, for the explant of condyles.

All femurs were dissected (operated and non-operated animal side), cleaned from soft tissues for histomorphometric measurements.

(b) *Histomorphometry: A*rticular cartilage of treated and control femoral condyles was examined macroscopically, after which the right femoral condyles were fixed in 4% buffered formalin, dehydrated and embedded in polymethylmethacrylate for histological evaluation. Sections of 20 µm in thickness were prepared (diamond saw rotating microtome Leica 1600, Leica spa, MI, I) from the center of the repaired area and stained with toluidine blue (Sigma Chemical Co. St Louis MD). Staining was performed in thermostat at 37°C for 20 min. Sections were carefully washed in water and a quick passage in 95% and 100% ethanol was performed before mounting with DPX mountant (Fluka BioChemika, Germany). A total of 3 serial central slices were analyzed for each knee by a blind-investigator, using a Zeiss Axioscope microscope (C.Zeiss, Oberkochen, Germany) to evaluate reparative cartilage following the Driscoll modified score [16]. A score was assigned to cell morphology, matrix staining, defect filling and surface regularity, with a total score of "perfect" repair or normal articular cartilage equal to 12, derived from the following characteristics of newly formed cartilage and defect healing: normal morphology, 4; normal matrix staining, 4; 100% filling of defect, 2; regular and smooth surface, 2. All the articular surface was analysed at a magnification of 5× (each area of 2.3 mm^2).

2.2.1.6 Statistical analysis Statistical analysis was performed using software package SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data are reported as mean ± standard deviations (SD) and the significance level was set at $P < 0.05$. After checking for normal distribution and homogeneity of variances, a one-way analysis of variance (ANOVA) was used to assess significance

3 Results and discussion

Thixotropic behavior: CMCA and Hyal hydrogels showed thixotropic properties, i.e., under an appropriate mechanical stimulus, they were able to change, although temporarily, their mechanical properties. Once removed the mechanical stimulus, they resumed the original consistence. This pointed out the property of these hydrogels to be injectable [17].

All the above is demonstrated and confirmed by the rheological analysis. In Fig. 1, the trend of the two rheological parameters G′ and G″ was reported for 100% CMCA and Hyal hydrogels. Increasing the oscillation stress from 0 to 10000 Pa, G′ and G″ decreased. Once a particular stress value was reached, a gel-sol transition was observed for both hydrogels. At an applied stress of about 800 Pa, the 100% Hyal hydrogel changed its consistence, showing G′ lower than G″, whereas 4000Pa are necessary to obtain a similar phenomenon on 100% CMCA hydrogel. Once removed the mechanical stimulation, an immediate recovery of the original hydrogel consistence was recorded. In fact, both G′ and G″ resumed their initial values, with G′ greater than G″ (Fig. 1a, b).

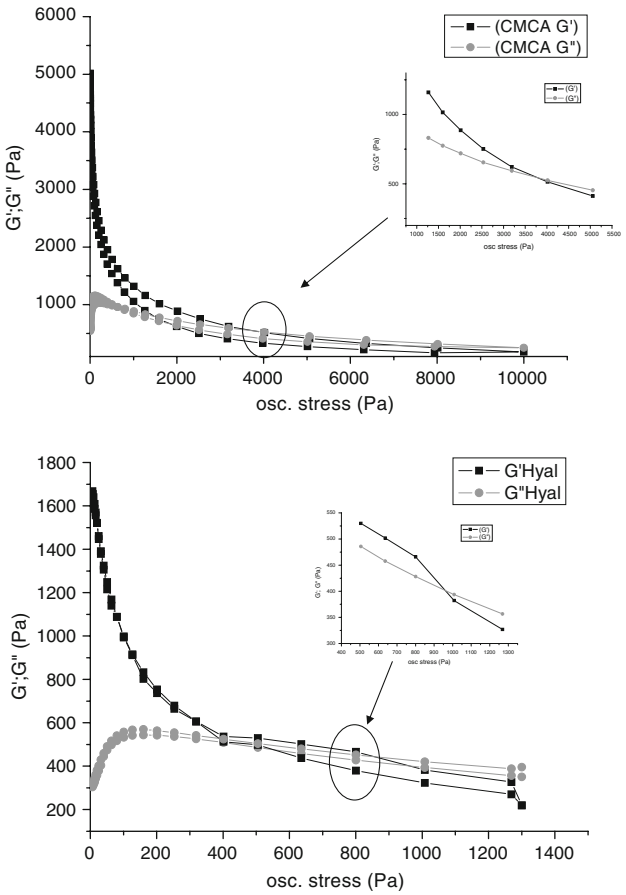

Fig. 1 Storage (G′) and loss (G″) moduli trend of CMCA (G′– and G″–) and Hyal hydrogel (G′, and G″,) as a function of increasing / decreasing stress

3.1 Cell proliferation and synthetic activity

The capability of the 100% CMCA hydrogel to stimulate chondrocyte proliferation and metabolic activity was compared with a 100% Hyal-based hydrogel whose activity towards chondrocytes is well known [9, 10]. Samples were incubated with NHAC and the cell proliferation and metabolic activity were tested after 7 and 14 days. The results of the WST1 test showed that chondrocytes proliferated in both CMCA and Hyal hydrogels without statistical differences (Fig. 2a, 14 days). Furthermore, chondrocytes seeded on CMCA and Hyal hydrogels maintained their phenotype as indicated by cathepsin B values. Actually, cathepsin B, which can be used as a soluble marker to evaluate the phenotype of cultured chondrocytes, is known to be produced by chondrocytes grown in a monolayer culture, while decreasing significantly when cells are cultured on substrates that promote a re-differentiation process [12, 13]. Cathepsin B results showed a significant decrease in hydrogel groups from days 7 to 14, indicating that chondrocytes were well differentiated during cell culture. As expected, the CTR group grown on polystyrene maintained the same level of Cathepsin B in both experimental times, this being an index of no differentiation (Fig. 2b, 14 days: CTR versus CMCA, Hyal: $P < 0.01$).

To verify chondrocyte anabolic activity, the production of collagen type II (CPII) and proteoglycans (PG) were evaluated (Fig. 2c, d). Chondrocytes cultured on hydrogels showed significantly higher values of CPII (CMCA, Hyal versus CTR, $P < 0.0001$) and PG (CMCA versus CTR, $P < 0.005$; Hyal versus CTR, $P < 0.0001$) in comparison with CTR on plate polystyrene culture.

The production of MMP1 and MMP13, collagenases involved in Type II collagen degradation, by chondrocytes seeded on CMCA and Hyal hydrogels was evaluated and compared with that of CRT. As shown in the graphs reported in Fig. 2e, f, chondrocytes seeded on CMCA and Hyal hydrogels showed no differences of MMP1 release when compared to CTR. On the contrary CMCA values were significantly lower in comparison with Hyal group ($P < 0.05$).

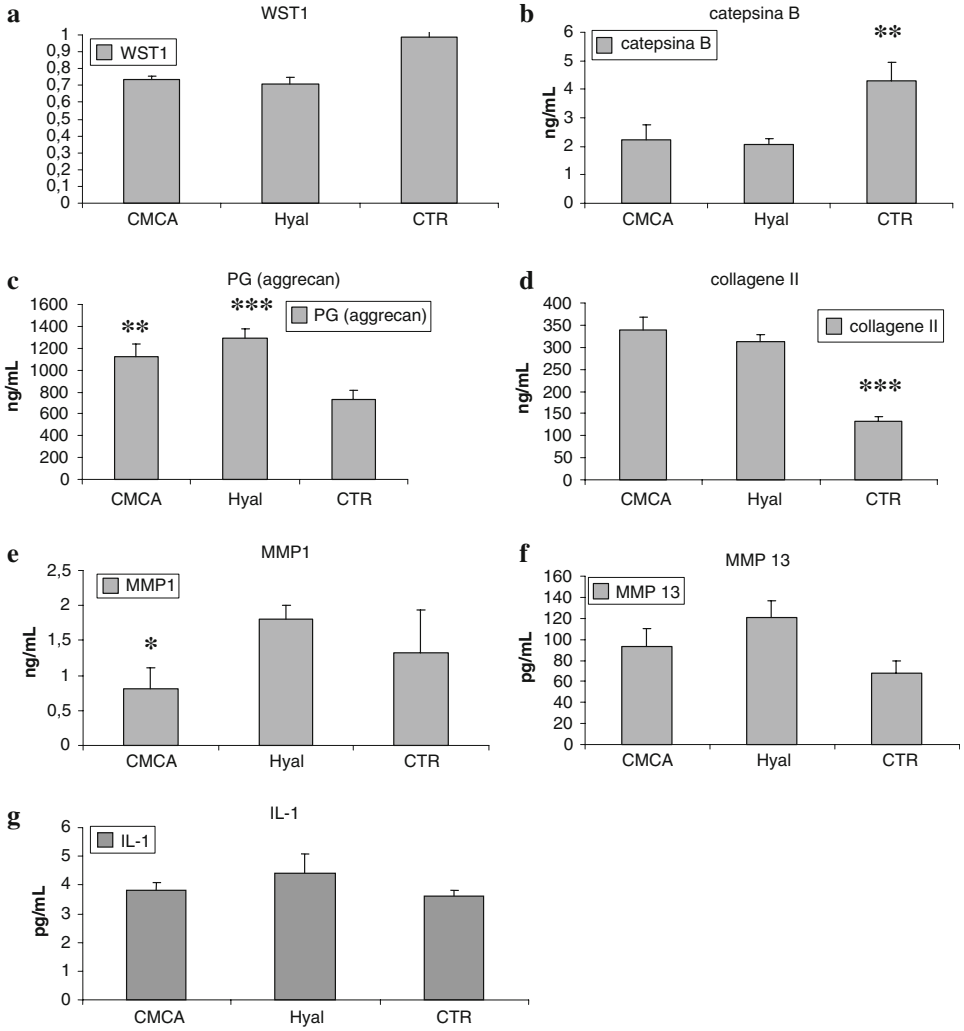

Fig. 2 Proliferation and activity of chondrocyte cultures (controls, CMCA and Hyal) after 14 days (Mean ± SD, $n = 6$) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$)

MMP13 did not show significative differences among all groups.

Finally, the inflammatory action of the hydrogel was evaluated by monitoring the release of IL-1. As shown in the graph reported in Fig. 2g, after 14 days no significant difference was found between the IL-1 release observed in the CTR and that in the presence of hydrogel.

3.1.1 Discussion of in vitro results

In vitro results suggested that CMCA ameliorated the chondrocyte differentiation as well as the synthesis of ECM components with respect to CRT, exhibiting a behavior similar to Hyal hydrogel that is considered the optimal material for OA therapy [9]. Actually, the

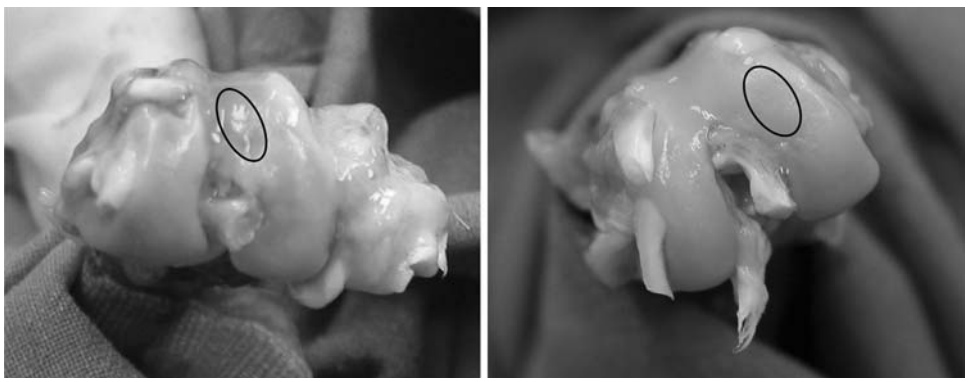

Fig. 3 Macroscopic photographs of the chondral lesion after 50 days: (**a**) Control defect; (**b**) CMCA treated defect

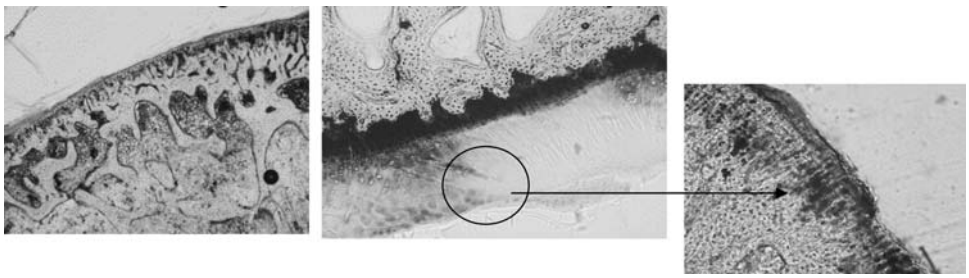

Fig. 4 histological sections from the chondral defect after 50 d days: (**a**) control defect: no tissue regeneration; (**b**) CMCA treated defect: mixed hyaline and fibrous new tissue; (**c**) columnar formations of chondrocytes dipped into a hyaline-like matrix. (Toluidine Blue staining; Magnification: 5×)

chondrocytes seeded on CMCA hydrogel showed a significant increased aggrecan production. Aggrecan is the characteristic proteoglycan that defines hyaline cartilage [12]. Aggrecan increase is important because it is responsible for the maintenance of the integrity of cartilage structure, providing unique biomechanical properties to the tissue. Also in terms of type II collagen production, CMCA showed a good performance, significantly increasing the production of this ECM component that provides structure and tensile strength to cartilage. CMCA hydrogel did not stimulate the release of MMP1 and MMP13, collagenases which target matrix components for degradation and are the only enzymes capable of degrading collagen in physiologic conditions [12]. So, on the basis of the good

Table 1 Histological grading scale for the evaluation of the articular cartilage repair

Grading scale (max 12p)	Points	30 days			50 days		
		Control	Hyal 100%	CMCA 100%	Control	Hyal 100%	CMCA 100%
Cell morphology							
Normal	4						
Mostly hyaline cartilage	3						
Mixed hyaline and fibrocartilage	2		2	2		4	2
Mostly fibrocartilage	1		2	2			2
Some fibrocartilage, mostly nonchondrocytic cells	0	4			4		
Mean		0	1.5	1.5	0	2.0	1.5
Matrix staining							
Normal	4						
Slightly reduced	3					1	2
reduced	2			1		2	1
Significantly reduced	1		4	3		1	1
No staining	0	4			4		
Mean		0	1.0	1.25	0	2.0	2.25
Filling of defect							
±100%	2					1	3
±50%	1		2	3		3	1
No fill	0	4	2	1	4		
Mean		0	0.5	0.75	0	1.25	1.75
Surface regularity							
Regular, smooth	2					2	1
Slightly irregular	1		4	2	2	2	2
irregular	0	4		2	2		1
Mean		0	1	0.5	0.5	1.5	1
Total mean score for each group		0.0	4.0	4.0	0.5	6.8	6.5

rheological performance and the optimal action towards normal human chondrocytes, CMCA hydrogel can be considered a potentially good filler cartilage defects.

Since the aim of this study was to find a substitute of Hyal-based hydrogel in the treatment of OA, the effect of this new hydrogel has been evaluated in vivo.

3.1.2 In vivo study

In all the animals, the wounds healed without complication, and no edema nor inflammation of the treated articulations were observed. At explant, gross examination of untreated condyles showed that, both after 30 and 50 days, the surface lesion appeared well visible because of a rough and opaque area. In treated condyle groups, after 30 days the defect appeared partially filled with a smoothed, slight irregular tissue, and after 50 days the area of the initial defect was less clearly definable from the adjacent intact cartilage (Fig. 3).

At the histomorphometric analysis, as expected, tissue healing was not observed in untreated lesions, either after 30 or 50 days (mean score: 0 at 30 days, and 0.5 at 50 days), although a thin and irregular layer of fibrous tissue was observed only after 50 days.

At 30 days, in the group treated with CMCA hydrogel the defect was not completely filled, and the presence of a slightly irregular layer of mostly fibrocartilaginous tissue was observed along the surface of the lesion. In all samples matrix staining was found to be reduced. The Driscoll modified score [16] of the histological grading scale was observed to vary from 2 to 6.

At 50 days, samples from the group treated with CMCA hydrogel showed a layer of mixed fibro cartilaginous and hyaline-like tissue. The defect was completely filled in 3 out of 4 samples, their surface being regular and smooth (Fig. 4). The staining of the matrix was only slightly reduced, while cluster and columnar formations of chondrocytes were observed in the new hyaline-like cartilage. The Driscoll modified score [16] ranged from 3 to 9.

The local treatment with CMCA improved the healing of cartilage lesions, showing a more regular filling of the defect and significantly higher values for the Driscoll modified score, when compared with untreated samples. Data of the present study were compared to previous ones obtained when using Hyal [9, 10] and, as shown by the values reported in Table 1, a very similar behavior between CMCA and Hyal hydrogels was observed.

4 Conclusions

Cartilage tissue engineering has been widely proposed as a method to repair injured or diseased cartilage. Most approaches to cartilage engineering involve delivery of cells via biodegradable scaffolds to regenerate tissue [18, 19]. Neverthless, the time necessary to the cells for colonizing and developing a new tissue is not comparable with the time necessary for hydrogel degradation. One of the most utilised materials for cartilage regeneration is alginate hydrogels because alginate supports chondrocytes and is biocompatible in delivering cells in human trials, however its degradation rate is too fast in comparison with cartilage regeneration [20]. A same trend has been found also with chondroitin-sulphate or Hyal based hydrogels. A cellulose derivative seems to overcame these disadvantages.

In vivo results obtained using the amidic derivative of carboxymethylcellulose in the form of hydrogel showed that intra-articular injection of CMCA stimulated cartilage healing, even if long-term studies are mandatory to exclude further tissue degeneration.

To conclude, on the basis of these preliminary results, CMCA can be ‘considered a potentially good filler cartilage defects in the OA therapy. Using CMCA hydrogel, we obtain an identical action in terms of regeneration of the tissue with respect to Hyal, but with a lower cost. The reduced degradation rate of CMCA in comparison with Hyal will allow fewer administrations and, consequently, reduce discomfort and the risk of infection for the patient.

Furthermore the thixotropy of CMCA hydrogel permits to this material to be included in the injectable hydrogel class which guarantee a minimally invasive and localised therapy.

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