MIXED TYPE I AND TYPE II COLLAGEN SCAFFOLD FOR CARTILAGE REPAIR: ULTRASTRUCTURAL STUDY OF SYNOVIAL MEMBRANE RESPONSE AND HEALING POTENTIAL *VERSUS* MICROFRACTURES (A PILOT STUDY)

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The association between microfracture of the subchondral plate and a coverage scaffold has emerged as a promising strategy to treat cartilage lesions in a one-step procedure. Between different types of scaffolds (e.g. collagen, hyaluronic acid, polyglycolic acid) currently studied, type I collagen scaffold is the most used for this purpose, and is currently adopted for humans. The aim of this study was to test a novel scaffold made of mixed type I and II collagen (I-IICS) in order to define the immunological reaction of the synovial tissue and the repair capabilities induced by the collagen membrane when associated with microfracture. Eight New Zealand White rabbits, aged 180 days, were operated on bilaterally on the medial femoral condyle. A circular cartilage lesion was performed up to the calcified layer of the medial femoral condyle, and the centre of the lesion was microfractured. Randomly, one of the two lesions was covered with the I-IICS (treated), and the other was left uncovered (control). The synovial membrane reaction and the quality of the cartilage tissue repair were investigated at 2, 90, 180 and 270 days macroscopically, histomorphologically and ultrastructurally. Expression of tumor necrosis factor-alpha (TNF-α) in synovial tissue by immunocytochemistry analyses was also investigated. In the control group, at 2 days gold particles were localized mainly on synoviocyte type A, less on synoviocytes type B and on collagen bundles; in the treated group the reaction is more intense in cells in the matrix, but at 180 days controls and treated joints were very similar. The synovial membranes of the joints receiving the I-IICS did not reveal significant changes compared to the age-matched controls. Signs of inflammation were present at the 90-day time-point, and became less evident at afterwards. The degradation of the scaffolds was already evident at the 90-day time-point. The quality of the cartilage repair of the rabbits treated with the I-IICS was slightly better in 5 cases out of 6 in comparison to the controls. However, a statistically significant difference was not detected (p=0.06). Scaffolds made of mixed type I and II collagen exhibited good biocompatibility properties *in vivo* and favored cartilage restoration when associated with microfracture, as shown in this pilot study.

Key words: cartilage, collagen type II, synovial membrane, immunogenicity, scaffold

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Articular cartilage lesions occur with an increasing frequency due to sport or automobile accidents. Defects of the articular cartilage do not repair adequately and often progress to cause osteoarthritis (1, 2). Recently, the association of microfracture of the subchondral plate and a coverage collagen scaffold has emerged as a promising strategy to treat cartilage lesions in a "one-step" procedure (3, 4). In this technique, microfracture permits the migration of mesenchymal stem cells (MSCs) from the subchondral bone and the scaffold should contain the clot in the defect and favor MSC differentiation towards the chondrocytic phenotype. It has already been shown that microfracture associated with bone marrow concentrate and a collagen type I membrane led to a good quality cartilage repair, comparable with the one obtained with the second generation autologous chondrocyte implantation (ACI) (3, 5).

Scaffolds which have been clinically used up to now in cartilage repair, are all composed of type I collagen which is not a specific component of the articular cartilage, but of all connective tissues. Since the principal matrix component of the articular cartilage is type II collagen, several investigators have proposed the advantageous properties of type II collagen scaffolds (6). *In vitro* studies suggest that mixed type I and type II or only type II collagen may be more effective than collage type I in favoring differentiation of MSCs from the bone marrow towards the chondrocytic phenotype (7-9). Again, *in vivo* studies on different animal models (sheep, rabbit, canine) have shown beneficial properties of type II collagen scaffolds in repairing knee cartilage defects (10-11).

However, in spite of these beneficial properties, the higher immunogenicity of the type II collagen compared with that of type I collagen appeared to be a drawback in the clinical application of type II collagen scaffolds. In fact, injections of type II collagen induced arthritis in rats (12), primates (13) and mice (14), and antibodies to type II collagen were shown play a major role in the initiation of the arthritic process (15) and their presence was detected in rheumatoid arthritis patients (16). Although type II collagen scaffolds have been tested in animal models without apparent adverse responses, none of these studies have been specifically designed to look for immunological reactions (8, 10, 11, 17) and in particular the proliferation of synovial cells and the infiltration of leukocytes in the synovial membrane that are fundamental events in the development of joint inflammation (18).

This study aims to (1) assess the morphostructural response of the synovial membrane to the implantation of a mixed type I and type II collagen scaffold (I-IICS) and (2) evaluate the cartilage repair potential of the I-IICS associated with microfracture in a rabbit model.

MATERIALS AND METHODS

Scaffold manufacturing

The collagen membrane of equine origin (Opocrin, Modena, Italy), consisted of a 75% type II collagen from articular cartilage and trachea and 25% type I collagen from the Achilles' tendon. Collagen was extracted and purified as previously described (19-20). Briefly, the fragments were immersed in an acidic solution containing pepsin at room temperature for 18 hours and then filtered. The solubilized collagen was precipitated by raising the pH up to neutrality and sterilized by filtration and precipitation with alkaline solutions. Salt removal was obtained through dialysis, and the fibers thus obtained were suspended with 0.3% acetic acid (BDH, VWR, Milan, Italy). The two types of collagen were mixed in a solution of NaOH (BDH, VWR, Milan, Italy) so that the molecules aggregated to form collagen fibrils. This mixture was placed in molds, dried at a constant temperature, and sterilized with gamma rays

Surgical procedure

Eight New Zealand white rabbits, aged 180 days and weighing about 4 Kg were anesthetized with ketamine and diazepam. A median skin incision and a medial parapatellar approach were carried out to expose the medial femoral condyle. A standardized circular lesion of the diameter of 4 mm (critical size defect) was performed at the center of the loading area of the medial femoral condyle, using a 4-millimeter drill guide. The lesion was brought up to the calcified layer of the cartilage. A small microfracture at the center of each defect was produced with a 21 G needle so as to obtain obvious bleeding from the subchondral bone. Even if microfracturing generally uses multiple fractures, in this experimental model the size of the cartilage lesion was not large enough to ensure the appropriate bleeding from the sub-chondral bone with multiple fractures. For this reason we chose to perform only one microfracture at the center of the lesion. To avoid the bias of loading

condition of the two condyles, one of the two lesions was covered randomly with the I-IICS that was press-fit within the lesion. The contralateral control lesion was left uncovered. Animals were housed separately under ethical protocol, and subjected to antibiotic therapy with penicillin and analgesia with buprenorphine. The sacrifice was carried out using an excess of anesthetic after a period of 2 (2 rabbits), 90 (2 rabbits), 180 (2 rabbits) and 270 (2 rabbits) days after intervention. The 2-day time-point was intended to confirm the presence of the membrane into the lesion site.

Synovial membrane morphological analyses
Light and electron microscopy

At 2, 90, 180 and 270 days after surgery, the knee joints were perfused with 4% paraformaldehyde (Sigma, St Louis, MO, USA) plus 0.5% glutaraldehyde (Fluka, Buchs, Switzerland) in PBS for 15 min. The synovial membrane was then carefully dissected and cut into three to four square millimeter pieces in order to obtain at least 14-21 tissue fragments for each experimental condition. Samples were fixed in 2.5% glutaraldehyde in PBS, post-fixed in 4% OsO_4 (Fluka, Buchs, Switzerland) in PBS, dehydrated and included in TLV low viscosity resin (TAAB, Aldermaston, UK). Semi-thin sections (0.8 μm thick) were obtained with an Ultracut ultramicrotome (Reickert-Jung, Wetzlar, Germany), stained with 1% Toluidine Blue-O (Sigma, St. Louis, Mo, USA) and observed by an Axiophot optical microscope (Carl Zeiss, Jena, Germany) allowing a detailed observation of the complex cellular environment of the synovial membrane.

Specimens of semi-thin synovial tissue were further subjected to morphometric analyses in order to better quantify structural changes. Only full-thickness samples (from the superficial lining to the sub-synovium) were used for morphometrical analysis according to parameters established in previous studies (21).

Parameters considered for the lining were: presence of cells (monolayer, bi-layer, multi-layer), arrangement of cells (aggregated, spread), cell shape (oval, spindle, dendritic), nucleus/cytoplasm ratio.

Parameters considered for the sub-intima and the sub-synovium were: cell type (fibroblasts, adipocytes), vessels (number, lumen width, basal membrane thickness), collagen content. To evaluate the frequency variations in number of the parameters considered in comparison to the controls, a series of optical micrographs (40-80) per time-point of treatment were taken for each specimen (14-21 specimens per animal) at the same low magnification (40 x), one micrograph for each frame. In this way, the number of cells was referred to the same surface area.

Ultra-thin sections (80 nm thick) of full-thickness synovial membranes were collected on copper grids, stained with uranyl acetate and lead citrate and observed under a transmission electron microscope Jeol 1200 EX (Jeol, Tokyo, Japan) operating at 80 kV.

Scanning electron microscopy

Specimens were fixed with 0.5% glutaraldehyde (Fluka, Buchs, Switzerland) in PBS pH7.3 for 30 min at room temperature. After washing in PBS, samples were observed with a Quanta 200 ESEM (FEI company, Eindhoven, Netherlands).

Immunocytochemistry

Specimens were fixed in 4% paraformaldehyde plus 0.5% glutaraldehyde, dehydrated and included in TAV as above, ultra-thin sections collected on nickel grids underwent immune-cytochemical analyses using Armenian hamster monoclonal antibody raised against purified recombinant mouse TNF-alpha (TNF-α), Santa Cruz Biotechnology, INC) for the evaluation of TNF-α expression.

Cartilage histological analysis

The femoral condyles of rabbits were fixed in neutral buffered formalin, decalcified in a solution of hydrochloric acid and formic acid, then included in paraffin and sectioned at a thickness of 5 μm in order to perform the histological analysis. Sections were stained with hematoxylin-eosin and safranin-O and histologically analyzed with an optical microscope (Leica Quantimet 500, Milan, Italy). The histological sections were evaluated with the modified O'Driscoll score (22).

Statistical analysis

Sinovial membrane analysis: Sections were examined by two independent investigators. For each time interval (2, 90, 180, 270 days after surgery) the back of the synovial membrane was cut into semi-thin sections and was observed at the same magnification (Object 40X) and at each time-point many micrographs (mean 41) were photographed in the same area along the lining. For each nominal variable a score from 0 to 3 (0=absent, 1=scarce, 2=moderately represented, 3=highly represented) was assigned while the vessels were counted numerically. Statistical significance was established with the Kruskall-Wallis and Bonferroni *t*-tests. The host response to the implanted scaffold is closely linked to individual variability, and for this reason the statistical analysis includes the pool of animals divided only for point in time (control *vs* implant, mean 82 observations).

Cartilage analysis: three independent observers blindly evaluated the different parameters of the O'Driscoll score. After discussion of the different results agreement on one value was taken as the final outcome. Data were analysed

with the Wilcoxon Exact Method for small samples test.

RESULTS

No rabbit showed signs of infective complications. At the end of the experiment all animals ambulated normally. No signs of pain or swelling or limpness were documented during the experimental period.

Macroscopical analysis

No sign of reactive synovitis or infection were detected at the time of sacrifice. The synovial fluid was clear in all the examined joints.

At the 2-day time-point the control defect was filled by a brownish blood clot which did not reach the level of the joint line (Fig. 1a), whilst the membrane remained press-fit within the lesion (Fig. 1b).

In both treated (Fig. $2a_1$, b_1, c_1 and d_1) and control condyles (Fig. $3a_1$, b_1, c_1 and d_1) at 90 and 180 days the repair tissue was easily distinguishable from the native cartilage, appearing whiter and more irregular than the surrounding tissue. A certain degree of cartilage degeneration next to the defect was detectable in about the half of the cases, without great differences between control and treated.

At the 270-day follow-up the lesion site was no more clearly detectable either in treated (Fig. $2e_1$ and f_1) or in control condyles (Fig. $3e_1$ and f_1), and different degrees of cartilage degeneration were seen. The cartilage surface in the control condyles appeared rough and uneven whilst in the treated condyles it appeared opaque. Overall, treated and control lesions showed a comparable degree of tissue filling.

Cartilage microscopic analysis

In the treated lesions, the modified O'Driscoll score ranged from 15 to 25 points, whilst in the control lesions it ranged from 10 to 19 points (Table I). A statistically significant difference in the quality of cartilage regeneration was not detected between the treated and control lesion for each animal ($p>0.05$). Also grouping together treated and control lesions, statistically significant variations of the O'Driscoll score were not detected at different time-points ($p>0.05$).

Treated lesions

At 90 days one case showed a mixture of fibrocartilage and hyaline-like cartilage with smooth surface and good metachromatic staining for safranin-O (Fig. $2a_{2,3}$). A focus of bone within the cartilage was noted (Fig. $2a_3$). The other case showed uneven tissue filling with critical boundaries with the surrounding cartilage, fissuring and massive

Table I. *Morphometric analyses on cartilage from control (C) and implanted (I) knees according to the O'Driscoll score (26).*

Follow-up	90 Days				180 Days				270 days			
Rabbits	C_1	I_1	C_2	I_2	C_1	I_1	C_2	I_2	C_1	I_1	C_2	I_2
Cellular morphology	4	4	2	4	4	4	4	4	4	4	2	4
Matrix staining (Safranin-O)	1	1	1	3	1	2	2	1	2	3	1	1
Surface regularity	3	2	2	3	2	3	1	2	3	3	1	3
Structural integrity	1	1	0	2	2	2	0	1	0	2	0	2
Thickness	2	2	2	2	1	2	0	2	2	2	1	1
Bonding	1	1	1	2	2	2	0	1	2	2	1	1
Hypocellularity	3	2	2	3	3	3	0	3	3	2	1	3
Clustering	0	0	1	2	1	2	0	0	0	1	0	0
Perilesional osteoartritis	2	2	2	3	3	3	3	3	2	3	3	2
TOTAL SCORE	**17**	**15**	**13**	**24**	**19**	**23**	**10**	**17**	**18**	**22**	**10**	**17**

Animals per group (C/I): 90 days (n=2); 180 days (n=2); 270 days (n=2)

Table II. *Morphometric analysis of the synovial membrane lining.*

Variables	Time points					
	2 Days		90-180 Days		270 Days	
	Controls	Implants	Controls	Implants	Controls	Implants
Mono/bi-layers	**2 ± 1.4**	**1.2 ± 1.5***	**3 ± 0.1**	**1.2 ± 1.5***	**1.6 ± 1.4**	**3.1 ± 1.1***
Multi-layers	2.1 ± 1.3	2.3 ± 1.3	**0.8 ± 1.3**	**2 ± 1.4***	**0.5 ± 0.9**	**1.2 ± 1.3***
Aggregated	2.5 ± 1.1	2.7 ± 0.8	3 ± 0.1	3 ± 0.1	**3 ± 0.1**	**2.6 ± 1***
Spread	0.7 ± 1.2	0.8 ± 1.2	0.3 ± 0.9	0.2 ± 0.7	**0.1 ± 0.1**	**0.9 ± 1.3***
Oval-shape	2.8 ± 0.7	3 ± 0.1	3 ± 0.1	3 ± 0.1	2.9 ± 0.3	3 ± 0.1
Spindle-shape	1.8 ± 1.4	1.9 ± 1.4	3 ± 0.1	3 ± 0.1	2.8 ± 0.8	3 ± 0.1
Dendritic-shape	1.7 ± 1.4	2 ± 1.3	0.6 ± 1.1	0.4 ± 1.3	0.1 ± 0.1	0.3 ± 0.8
Nucleus/cytoplasm ratio	0.9 ± 0.6	1 ± 0.6	**1 ± 0.8**	**1.2 ± 0.6***	0.5 ± 0.4	0.6 ± 0.5

*N° of Animals per group and observations: 2 days (n=2) observations 59 controls vs 52 implants; 90-180 days (n=4) observations 123 controls vs 101 implants; 270 days (n=2) observations 46 controls vs 42 implants; *p≤ 0.05 implants vs controls*

Table III. *Morphometric analysis of the synovial membrane sub-intima.*

Variables	Time points					
	2 Days		90-180 Days		270 Days	
	Controls	Implants	Controls	Implants	Controls	Implants
Fibroblasts	**1.6 ± 1.4**	**2.8 ± 0.8***	2 ± 1	1.9 ± 1.1	1.1 ± 1.4	0.7 ± 1.1
Adipocytes	**1.8 ± 1.4**	**0.7 ± 1.3***	**1.7 ± 1.4**	**1.2 ± 1.4***	2.6 ± 1	2.3 ± 1.3
Vessel number[1]	9.2 ± 6	12 ± 10.5	4.5 ± 4.4	5.3 ± 3.6	6.5 ± 5.7	4.7 ± 4.7
Vessel width	2.8 ± 0.7	2.7 ± 0.8	2.2 ± 1	2.4 ± 0.9	1.6 ± 0.9	1.4 ± 0.6
Vessel basement membrane thickness	**3 ± 1.1**	**0.5 ± 1.1***	0.9 ± 1.5	0.6 ± 1.2	0.2 ± 0.8	0.6 ± 1.2
Collagen	**1.9 ± 1.3**	**2.8 ± 0.7***	2 ± 0.9	1.8 ± 1.2	1.2 ± 1.4	0.6 ± 1.2

*N° of Animals per group and observations: 2 days (n=2) observations 59 controls vs 52 implants; 90-180 days (n=4) observations 123 controls vs 101 implants; 270 days (n=2) observations 46 controls vs 42 implants; *p≤ 0.05 implants vs controls*

[1] *The number of blood vessels /μm² is reported*

clustering (Fig. $2b_{2\text{-}4}$).

At 180 days one case showed a thin fibrocartilage layer with fibrillated surface (Fig. $2c_2$), scarce metachromatic staining (Fig. $2c_3$), subchondral bone remodeling and sclerosis (Fig. $2c_4$). The other case showed a thick hyaline-like cartilage layer slightly elevated in respect to the native cartilage line, associated with subchondral bone remodeling (Fig. $2d_{2\text{-}4}$).

At 270 days one case showed hyaline-like cartilage with smooth surface and excellent metachromatic staining (Fig. $2e_{2\text{-}4}$). The other case showed fibrocartilage with scarce metachromatic staining (Fig. $2f_2$), marked perilesional cartilage degeneration (Fig. $2f_3$), abundant clustering and fibrillated surface (Fig. $2f_4$).

Control lesions

At 90 days both lesions showed good tissue filling, with a good cartilage surface (Fig. $3a_2$ and b_2). Cartilage matrix was mostly fibrocartilaginous

Table IV. *Morphometric analysis of the synovial membrane sub-synovium.*

Variables	Time points					
	2 Days		90-180 Days		270 Days	
	Controls	Implants	Controls	Implants	Controls	Implants
Fibroblasts	**0.8 ± 1.2**	**1.6 ± 1.5***	**0.1 ± 0.2**	**0.9 ± 1.1***	0.5 ± 1	0.3 ± 0.7
Adipocytes	2 ± 1.3	1.7 ± 1.4	2.9 ± 0.2	2.3 ± 1.1	3 ± 0.1	3 ± 0.1
Vessel number[1]	**6.1 ± 3**	**8.8 ± 9.9***	**2.6 ± 2.3**	**4.1 ±3.1***	**5.3 ± 2.5**	**3.5 ± 2.8***
Vessel width	2.8 ± 0.7	2.7 ± 0.8	2.3 ± 1.3	2.6 ± 0.9	**2.6 ± 0.6**	**2.2 ± 0.8***
Vessel basement membrane thickness	**0.3 ± 0.9**	**1.4 ± 1.6***	**0.4 ± 0.9**	**0.7 ± 1.2***	0.6 ± 1.3	1.1 ± 1.5
Collagen	1.2 ± 1.4	1.7 ± 1.5	0.3 ± 0.9	0.9 ± 1.1	0.5 ± 1	0.3 ± 0.7

N° of Animals per group and observations: 2 days (n=2) observations 59 controls vs 52 implants; 90-180 days (n=4) observations 123 controls vs 101 implants; 270 days (n=2) observations 46 controls vs 42 implants;
**p≤ 0.05 implants vs controls.*
[1] The number of blood vessels/μm² is reported

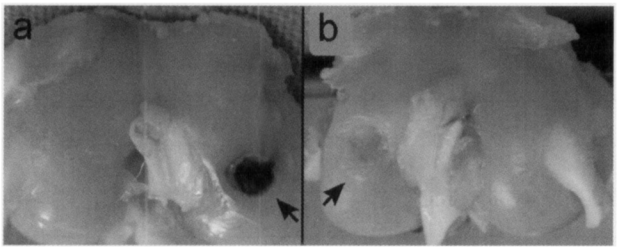

Fig. 1. *Macroscopic analysis of the cartilage lesion 2 days post-operatively:* ***a)*** *control lesion showing the blood clot;* ***b)*** *treated lesion showing the I-IICS in place.*

with scarce metachromatic staining for safranin-O (Fig. $3a_3$ and $b_{3,4}$). Osteochondral junction and the boundary between the lesion and the surrounding cartilage were critical in one case (Fig. $3a_{3,4}$). In the same case native cartilage fibrillation was visible (Fig. $3a_2$).

At 180 days an extreme variability was observed. In one case reparative tissue was partly disrupted (Fig. $3c_{2,3}$), and in the other case there was abundant cartilage formation well below the line of the osteochondral junction of the native cartilage (Fig. $3d_2$). A focus of ossification within the cartilage (Fig. $3d_{3,4}$) and massive clustering(Fig. $3d_5$) were also observed.

At 270 days, fibrocartilage formation with a smooth surface and good metachromatic staining for Safranin-O was observed in one case (Fig. $3e_2$). The same sample showed diminished structural integrity just above the osteochondral junction (Fig. $3e_{3,4}$). The other case showed incomplete tissue filling, cartilage fissuring and scarce metachromatic staining for safranin-O (Fig. $3f_{2,3}$). Foci of cartilage within the subchondral bone were also observed (Fig. $3f_{2,4}$). At all the time-points a tendency to subchondral bone remodeling and fusion of the trabeculae was observed.

Synovial membrane microscopical analysis

Two days after surgery, both in control (Fig. 4a and c) and in implanted knees (Fig. 4b and d), the synovium showed a moderate oedema and numerous vessels, as expected in an injured tissue. Consistently, the synovial lining showed an hypercellularity, with a large number of oval and spindle-shaped cells resembling type A synoviocytes, characterized by cytoplasmic extrusions, numerous lysosomes and few mitochondria. By contrast, type B synoviocytes were less abundant, exhibited a well developed endoplasmic reticulum and Golgi apparatus, as indicators of a synthetic phenotype (Fig. 5a and b). Synoviocytes appeared mainly organized in multilayers, especially in samples from knees hosting the collagen scaffold (Table II). In the sub-intima and in subsynovium of the same specimens, fibroblasts were rather numerous whereas adipocytes were scarce (Tables III and IV). Vascularization appeared significantly more pronounced in the sub-synovium of implanted compared to control specimens (Table V). Only in rare and isolated samples polymorphonuclear leukocytes and mastzellen were identified. These

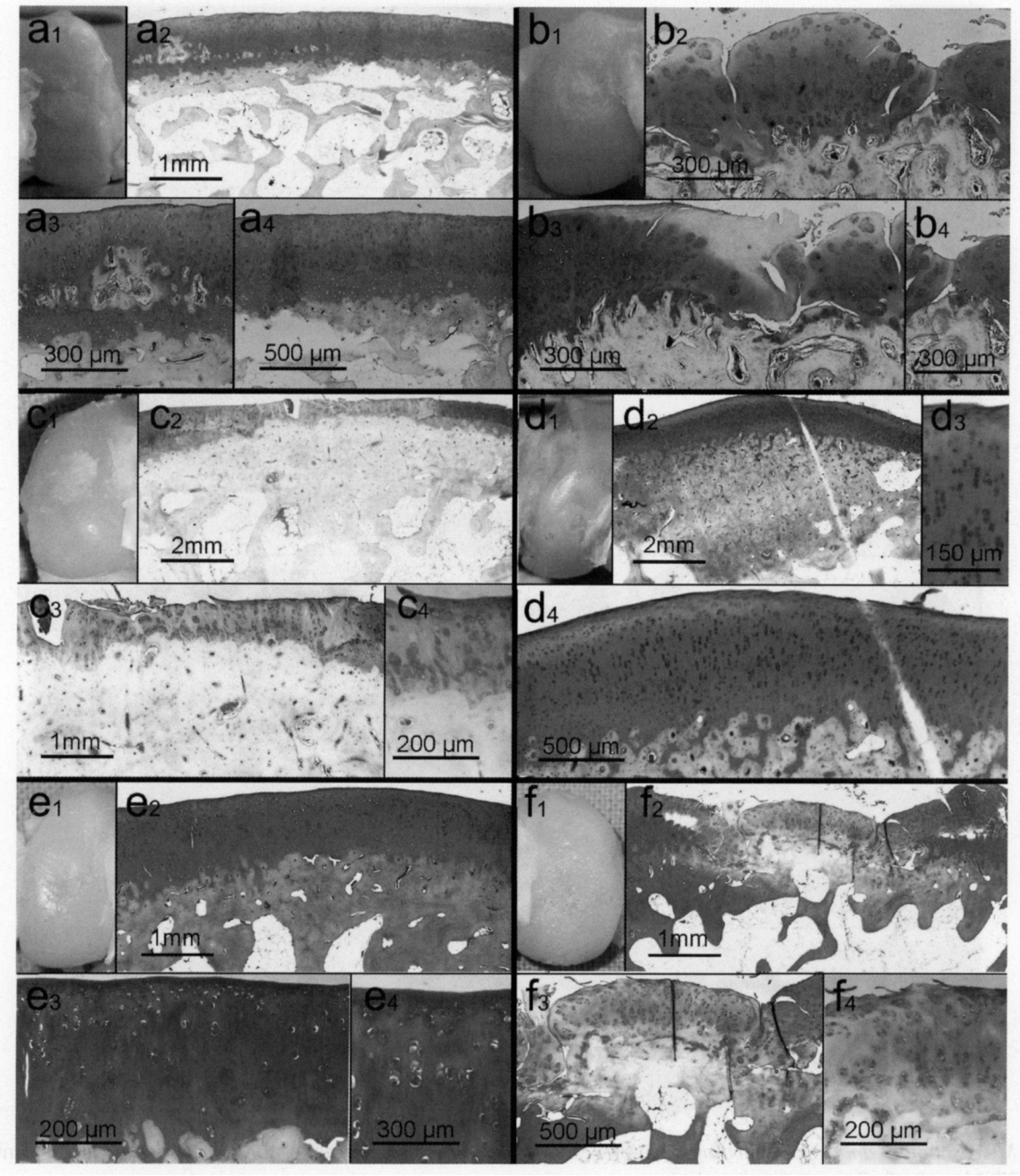

Fig. 2. *Microscopical analysis of cartilage lesions treated with microfracture and I-IICS implantation at 90 days (**a**, **b**), at 180 days (**c**, **d**) and at 270 days (**e** and **f**). See text for description.*

two aspects are indicative of a slight inflammatory reaction that invests the deep parts of the synovial membrane, parts that have been damaged most likely after surgery. During the first days after surgery, in animals with implanted scaffolds (Fig. 6b), slight signs of inflammation were associated with TNF-α expression.

In control specimens, at 90-180 days after surgery, the synovial membrane exhibited a clear evidence of tissue remodeling (Fig. 7a). The synovial lining had a lower number of cells compared to day 2 post-surgery, and mostly organized in few layers. At 270 days after surgery, the organization of the tissue appeared rather stabilized, with the majority of

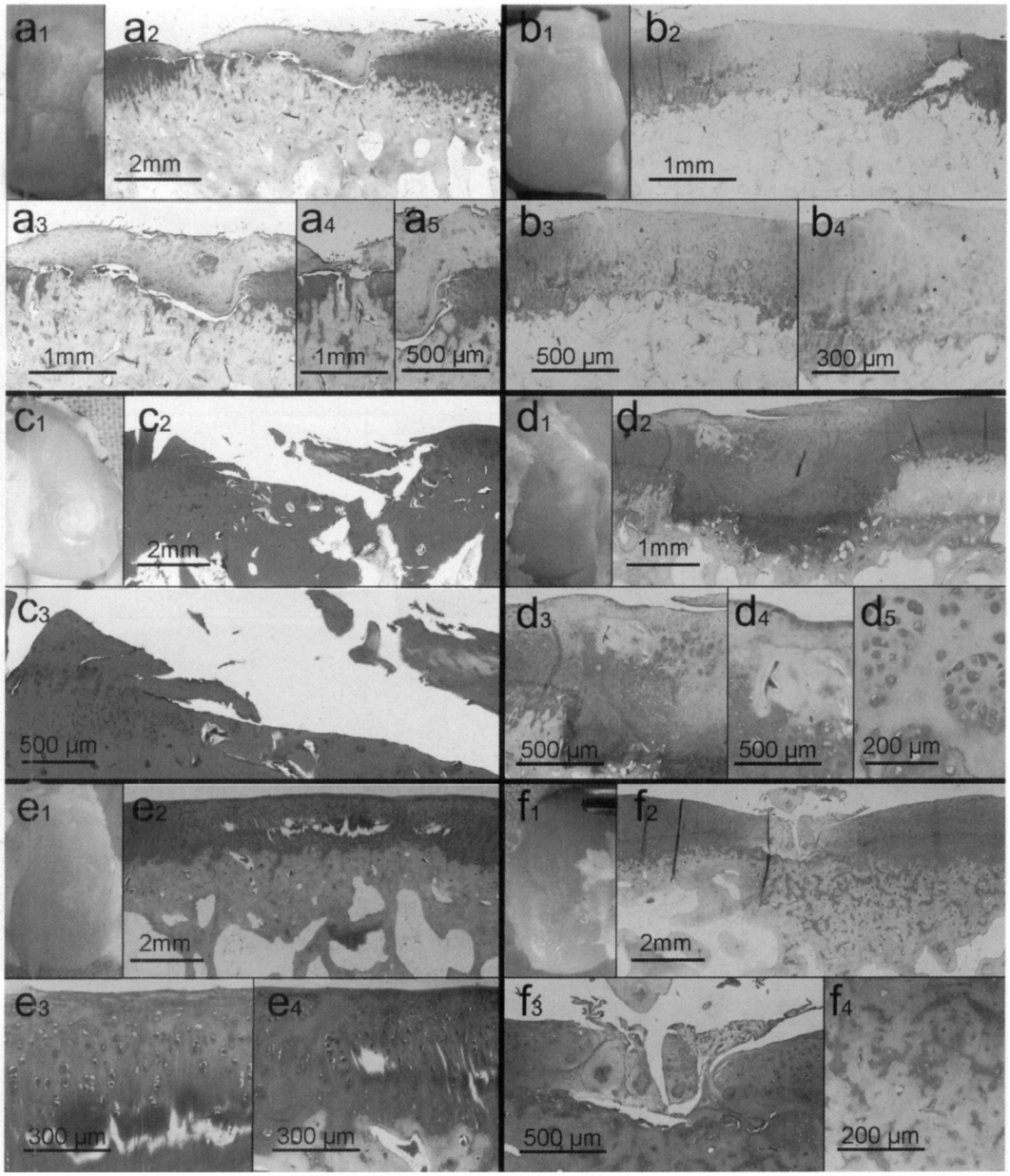

Fig. 3. *Microscopical analysis of cartilage control lesions (uncovered microfracture) at 90 days (**a**, **b**), at 180 days (**c**, **d**) and at 270 days (**e**, **f**). See text for description.*

synoviocytes increasing their cell-cell contacts and forming mono or bilayers (Fig. 8a).

Vascularization was further reduced compared with previous times and tissue remodelling did not lead to a fibrotic process, since the amount and organization of collagen bundles were similar to those of a normal synovium (Tables III and IV).

In samples hosting the implanted collagen scaffold, as already mentioned, synoviocytes were mainly organized into multi-layers compared to control specimens at 2 days after surgery. At 90-180 days, synoviocytes in the superficial lining were mainly organized into bi-layers (Fig. 7b) and, at the ultrastructural level, type A synoviocytes showed numerous lysosomes, filled with electron-dense material, suggesting degradation of the collagen scaffold (Fig. 7d). In the sub-intima the extracellular matrix was comprised of small collagen bundles and

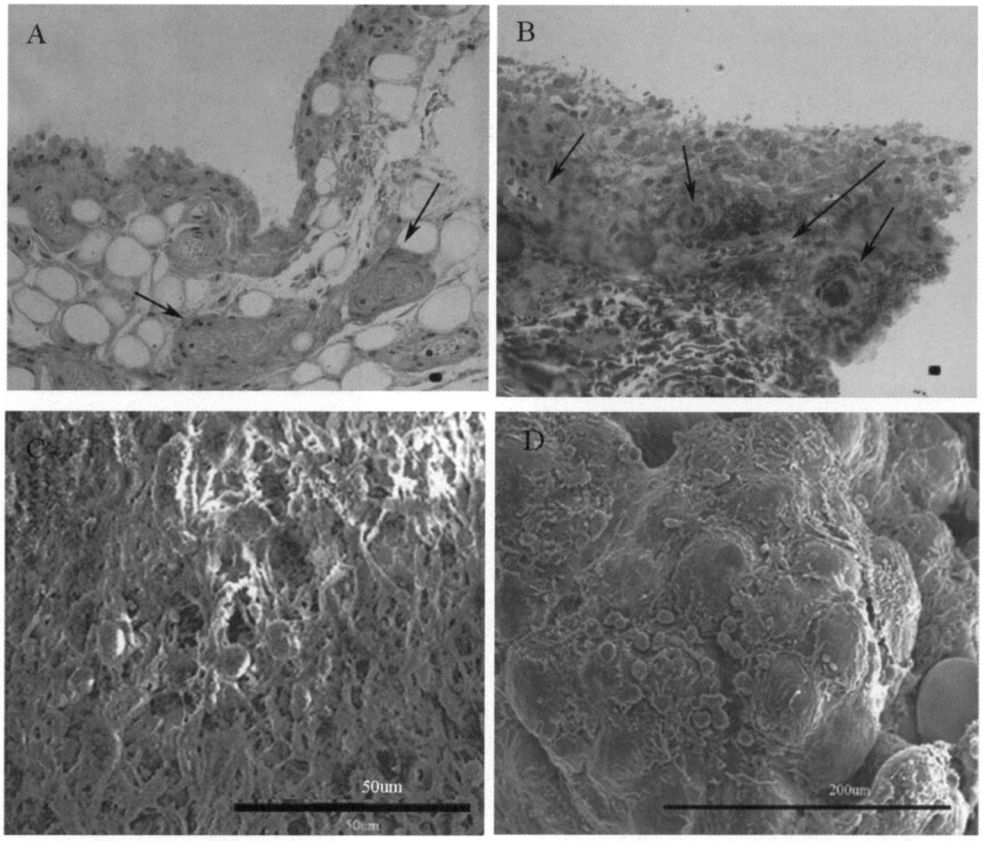

Fig. 4. *Optical (**a, b**) and scanning ultrastructure (**c, d**) analysis of the synovial membrane of control (**a, c**) and treated rabbits (**b, d**) 2 days post-operatively. a, c) Synovial lining is made by bi-layers, the vascular component is well represented both in the sub-lining and in sub-synovial lining (arrow), the synovial membrane is of adipose type; b, d) the complex organization of the cellular lining with areas of multilayer can be appreciated. Optical (Bar = 1m). ESEM (Bar= 50-200m).*

numerous elastic fibres (Fig. 7c). At 270 days after surgery, a slightly higher cellularity compared to controls (Fig. 8b) was still observed in the synovial lining, although cells appeared mainly organized in mono/bi-layers and intercellular contacts were re-established (Fig. 8b). In the sub-intima and in the sub-synovium, fibroblasts and blood vessels were reduced compared to control specimens (Tables III and IV).

DISCUSSION

Despite articular cartilage extracellular matrix being mainly composed by type II collagen, only a few *in vivo* animal studies adopted type II collagen scaffolds to experimentally treat cartilage defects, and none of them looked for type II collagen-induced inflammatory reactions and synovial membrane morphological changes (8, 10, 11, 17, 23). The reactivity of the synovial membrane is of paramount importance for the restoration of joint homeostasis, as it has been reported that, besides the bone marrow, the synovial membrane (24) and the mesenchymal perivascular niche (25) represent a valuable cell sources for cartilage repair, further emphasizing the active role of the synovium within the joint cavity. We evaluated the presence of anti-TNF-α because it is the first cytokine to appear after an inflammatory stimulus and stimulates the secretion of other inflammatory cytokines.

Moreover, several authors have suggested that in rheumatoid arthritis, in which both tissues, cartilage and synovium, are studied, IL-1 controls especially the cartilage damage and bone resorption, while

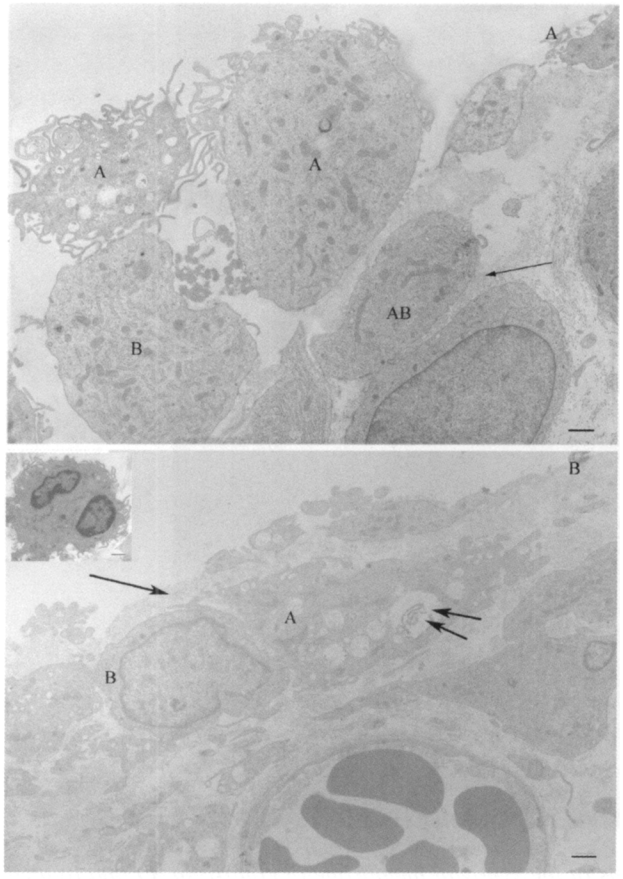

Fig. 5. *Ultrastructure of the synovial membrane 2 days post-operatively. In control (**a**) B synoviocytes have traits of pericellular coat still evident (arrow). In the treated group (**b**) the cell hyperplasia of type A, B, and AB synoviocytes, the presence of mitosis (insert) and oedema are characteristic of an inflammatory reaction.(Bar = 1m).*

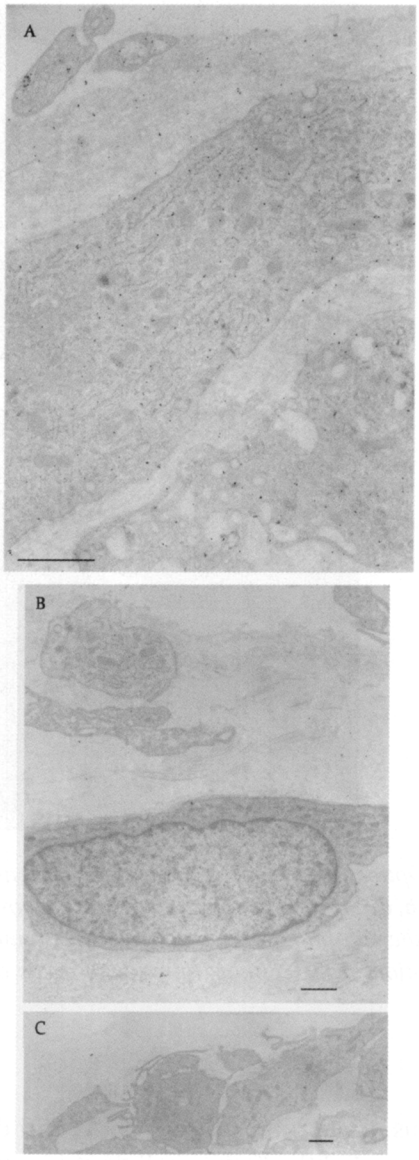

Fig. 6. *Immunocytochemistry: TNF-α expression in the rabbit synovial membrane 2 days post-operatively. **a)** In the control group gold particles are localized mainly on synoviocytes type A, less on synoviocytes type B and on collagen bundles; **b)** In the treated group a weak immunostaining for TNF-α is observed in the tissue; **c)** Negative control of reaction Bar = 1m)*

TNF-α controls mainly the inflammatory process, and at high levels can increase matrix metalloproteinase synthesis with tissue damage (26, 27)

This study aimed to firstly assess the morphostructural response of the synovial membrane to the implantation of the I-IICS and, secondly, to evaluate the cartilage repair potential of the I-IICS associated with microfracture in the rabbit model.

The matrix tested in the present study consisted of a mixture of type I and II collagen. The rationale behind this specific composition is that type II collagen stimulates the chondrocytic differentiation of MSCs (7) and the mechanically more stable type-I collagen provides adequate coverage to the bone marrow blood clot (10).

The structural organization of synovial membranes in controls was generally maintained, although the surgical procedure, per se, induced a moderate inflammatory response. In the treated group the examined parameters indicated cell hyperplasia in the lining with the disappearance of

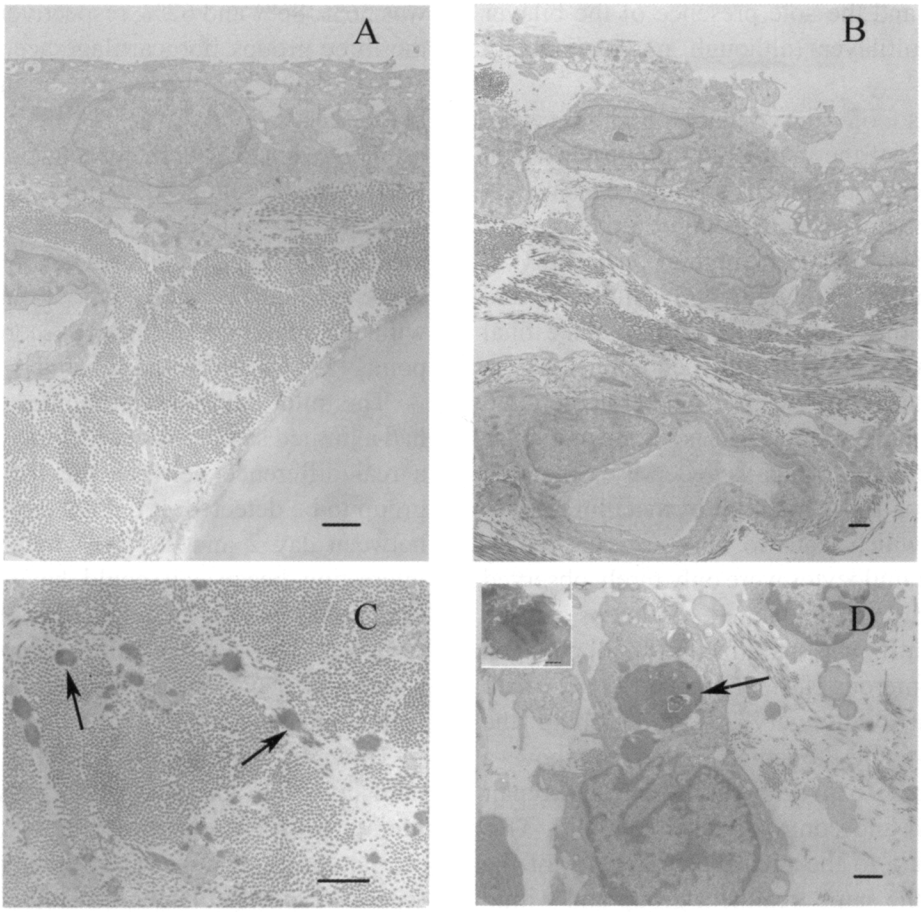

Fig. 7. *Ultrastructure of the synovial membrane 90-180 days post-operatively. Inflammatory reaction is not evident either in the controls (**a, c**) or in the treated (**b, d**). In this latter group, cells secrete proteins and capillaries are dilated (b). Lysosomes contained in type A synoviocytes are rich in degraded material (d) (arrow), probably fragments of the scaffold (insert) present in the extracellular matrix. In the extracellular matrix (c) there are elastic fibers (arrow) and normal collagen (Bar = 1m).*

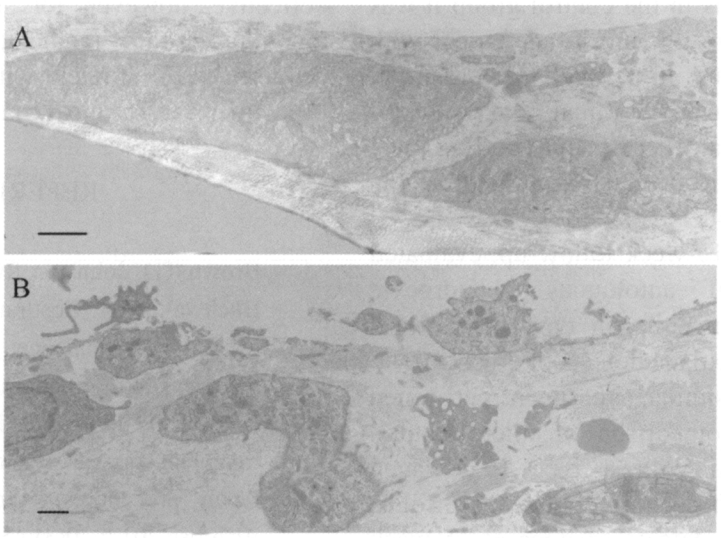

Fig. 8. *At 270 days there is no appreciable difference between controls (**a**) and treated (**b**) - in both, cells are arranged predominantly in monolayer and collagen fibrils are in contact with the synovial fluid.*

the monolayer and the sole presence of the bilayer and of the multilayer (although not statistically significantly).

The results of this study highlight the biocompatibility of the matrix scaffold used, which was progressively degraded without causing an inflammatory response. Interestingly, in implanted specimens, the synovial membrane, especially at earlier time-points, exhibited high cellularity and vascularization that reverted to normal at the final point of follow-up. It is worth mentioning that in these specimens, despite the high number of recruited cells, the synovial membrane was not a site of intense inflammatory process, which is generally associated with increased matrix degradation by metalloproeases and elastases, which leads to fibrotic tissue repair. Consistently, granulocytes were only rarely observed in the synovium from implanted specimens and the extracellular matrix was organized in small collagen bundles and numerous elastic fibres.

The present preliminary study, grouping the different time-points together, failed to show a significant difference in the quality of cartilage repair between treated and control groups ($p>0.05$). This was possibly due to the great heterogeneity of the data, even for the same treatment group and within the same time-point (Fig. 2 and 3). However, a trend towards a better quality of the repair tissue was shown for the group implanted with the I-IICS. In the treated group, hyaline-like cartilage alone or mixed with fibrocartilage (Fig. 2) was observed in 50% of the specimens, whereas in the control group it was never present. These results are in agreement with previously reported data. In particular, Giordano et al. have recently shown a better quality of the repair tissue with subchondral microfracture and a type I collagen membrane in respect to microfracture alone in a growing sheep model (29).

Nherer et al., in a 15-week follow-up, evaluated the effectiveness of autologous chondrocyte implantation (ACI) on collagen type II scaffolds placed on trochlear cartilage defects of dogs (8).

Consistently, in a similar experiment performed by Breinan et al., comparison of microfracture, microfracture covered with a type II collagen scaffolds and ACI on type II collagen scaffolds, on trochlear cartilage defects of dogs at 15-week follow-up, revealed that the percentage of defect fill was 56%, 86% and 62%, respectively, and that in all the three groups fibrocartilage accounted for about 70% of defect fill, the balance being mostly fibrous tissue (23).

In agreement with the promising results obtained with type II collagen scaffolds, Funayama et al. evaluated the autologous chondrocyte implantation on a collagen type II collagen gel on 16 New Zealand white rabbits. The mean overall outcome evaluated with the modified O'Driscoll score was about 20 points (17), as in the present study.

This pilot study had a number of limitations. It had a limited sample size which may have prevented a real difference between the treated and control group to be detected. It did not explore a time-point between day 2 and day 90 which may have been interesting because it could have represented the initial phase of the healing and of the inflammatory response. The synovial fluid composition was not assessed, because priority was given to the synovial membrane analyses.

However, this study has also a few strong points: to our knowledge it is the first study in literature which looked at the synovial membrane morphological changes after the implant of a mixed type I - type II collagen scaffold. It examined the inflammatory response thoroughly analyzing the membrane histologically, morphologically and with immunohistochemistry.

The use of mixed type I and type II collagen membrane has the potential to be safe and effective, however, for showing an improvement in microfracture mediated cartilage repair further experimental studies with a larger number of specimens and clinical trials are required.

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