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## Comparison of the therapeutic effect of amniotic fluid stem cells and their exosomes on monoiodoacetate-induced animal model of osteoarthritis <<Query: Title has been modified as per journals – please check. Ans: The title is ok.>>

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## Abstract

The cartilage tissue engineering associated with stem cell-related therapies is becoming very interesting since adult articular cartilage has limited intrinsic capacity for regeneration upon injury. Amniotic fluid stem cells (AFSC) have been shown to produce exosomes with growth factors and immunomodulating molecules that could stop tissue degradation and induce cartilage repair. Based on this state of the art, the main aim of this study was to explore the efficacy of the secreted exosomes, compared to their AFSC source, in MIA-induced animal model of osteoarthritis mimicking a chronic and degenerative process, where inflammation is also involved and lead to irreversible joint damage. Exosomes, obtained by the use of a commercial kit, prior to the injection in animal knee joints, were characterized for the presence of typical markers and HGF, TGF $\beta$ , and IDO. Then, analyses were performed by histology, immunohistochemistry, and behavioral scoring up to 3 weeks after the treatment. Exosome-treated defects showed enhanced pain tolerance level and improved histological scores than the AFSC-treated defects. Indeed by 3 weeks, TGF $\beta$ -rich exosome samples induced an almost complete restoration of cartilage with good surface regularity and with the characteristic of hyaline cartilage. Moreover, cells positive for resolving macrophage marker were more easily detectable into exosome-treated joints. Therefore, a modulating role for exosomes on macrophage polarization is conceivable, as demonstrated also by experiments performed on THP1 macrophages. In conclusion, this study demonstrates for the first time the efficacy of human AFSC exosomes in counteract cartilage damage, showing a positive correlation with their TGF $\beta$  content.

Keywords: exosomes; osteoarthritis; stem cells

**Figure S1.** Experimental in vivo design from time 0 to week 6.

**Figure S2.** Collagen II and Sox9 staining in control and MIA samples. ~~XXX~~.

**Figure S3.** ~~XXX~~. iNOS and arginase staining in control and MIA samples.

## Abbreviations

AFSC	amniotic fluid stem cells
BSA	bovine serum albumin
DABCO	1,4-diazabicyclo(2.2.2)octane
DAPI	4',6-diamidino-2-phenylindole
EDTA	ethylenediaminetetraacetic acid
EXO	exosomes
GAG	glycosaminoglycan
H&E	hematoxylin and eosin
HGF	hepatocyte growth factor
IDO	indoleamine 2,3-dioxygenase
IF	immunofluorescence
MIA	monoiodoacetate
OA	osteoarthritis
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
SE	standard error
TBS	tris-buffered saline
TGF $\beta$	transforming growth factor beta
TxTBS	Triton-X-100 TBS
WB	western blot

## 1 INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease characterized by degenerative changes in articular cartilage and secondary bone hyperplasia. Because articular cartilage has a particular structure, without blood vessels and with a low conversion rate of chondrocytes in the cartilage matrix, the treatment shows numerous clinical challenges. Traditional OA treatment is primarily symptomatic with pain management, which cannot contribute to the regeneration of degenerated cartilage or the reduction of inflammation. Furthermore, the mixed fibrous cartilaginous tissue generated is not the same as the natural hyaline cartilage. Mesenchymal stem cells (MSCs) have become the most widely explored cell therapy in OA due to their ability to differentiate into chondrocytes and their immunomodulatory properties.[1] Several experiments done in animal models of knee OA have shown that MSC therapy may delay progressive degeneration of the joint.[2, 3] Most human studies support the notion that the short-term application of MSCs is safe and feasible; however, further experiments are needed. Importantly, we still need a clear evidence confirming efficacy of MSC transplantation in patients with OA.[4] Further randomized controlled trials are required to estimate the potential of MSCs in cartilage repair and to evaluate advantages and disadvantages of stem cell treatment.[5]

In recent years, most studies have suggested that MSCs mainly regulate local inflammation, apoptosis, and cell proliferation through the paracrine mechanism, rather than directly differentiate into chondrocytes to participate in the repair of tissues.[6] Indeed, it is noteworthy that MSC participate in the local immune regulation mechanism, suppressing T cell proliferation, influencing the polarization of macrophages and the secretion of antibodies.[7] The MSC secretome includes soluble factors and micro-vesicles such as exosomes that behave as communication vectors between cells, which mainly supply various mRNAs, microRNAs, DNA, proteins, lipids, and other bioactive substances.[8] In recent years, a growing number of researchers believe that even exosomes secreted by MSC could play a role in the treatment of OA[9] since they are also rich in proteins and extracellular matrix (ECM) enzymes, which regulate and restore ECM balance. Thus, exosomes

obtained from MSC will probably become the main modality of treatment for clinical OA, avoiding the injection of cells and the related issues.

In this scenario, amniotic fluid stem cells (AFSC) are defined as largely multipotent cells, positive for mesenchymal markers and can be widely expanded in culture. Moreover, they are not tumorigenic and can be easily cryopreserved in cellular banks. Considering the fact that amniotic fluid is obtained through routine prenatal diagnosis, with minimal invasive procedure and without ethical concerns, AFSCs represent a valuable source for cell-based therapy of organ-specific or systemic degenerative and inflammatory diseases. During the last decade, the therapeutic potential of AFSCs, based on their huge differentiation capacity and immunomodulatory characteristics, has been extensively explored in animal models of degenerative and inflammatory diseases.[10] We have recently demonstrated the immunomodulation ability of AFSC: at first we showed that hAFSCs not only are less immunogenic but also can secrete immunoregulatory factors that may be useful in autoimmune diseases or allogeneic implants,[11] then we studied in deep the immunomodulatory effect of hAFSC's exosomes on peripheral blood mononuclear cells.[12] Moreover, we clearly established that hAFSCs promote the repair and regeneration of skeletal tissues after transplantation in an animal model of critical size bone defects.[13, 14]

Basing on all these considerations, the main purpose of this study was to explore the efficacy of secreted exosomes, compared to their hAFS cell source, in an OA animal model. Therefore, OA was created on the knees of rats with monoiodoacetate (MIA) injection: chemical OA induction is easy, reproducible, eliminates the need for surgery, and avoids possible infection issues. MIA-induced OA model is regularly used to measure pain behavior and drug therapy to resolve the pain in animals such as mice and rats. This model may be more predictive of drug efficacy than other pain models used to test OA drugs.[15]

Then the defects were treated with exosomes or stem cells derived from four amniocenteses. After 10 days, treatment with exosomes was repeated, in order to make the treatment comparable with the one with cells, at least in part, engrafted and still producing the secretome. Indeed, this is the first study focused on the comparison between exosomes and adult stem cells. Analyses that have been performed up to 3 weeks post-treatment and post-mortem are behavioral (pain tolerance test) and histological/immunohistochemical, respectively.

## **2 MATERIAL AND METHODS**

### **2.1 Amniotic fluid collection**

The AFSC were obtained from four amniotic fluids collected from pregnant women (mean age  $35.7 \pm SD$  1.2) between the 16th and 17th weeks of gestation who underwent amniocentesis for the maternal request (not for fetal anomalies) at Policlinico Hospital of Modena. The amniocentesis was performed under continuous ultrasound guidance, in a sterile field, with 23-Gauge needles. The risks related to the procedure were explained to all patients before the invasive procedure and a signed consent was collected by the ob-gyn specialist (E.B.) before starting the exam. During this pre-amniocentesis interview, pregnant women were informed of the purpose of the study and the informed consents to the study were obtained in accordance with the Italian law and the guidelines of the ethics committee (protocol 360/2017 dated December 15, 2017 approved by Area Vasta Emilia Nord).

Supernumerary (unused) flask of AF cells cultured in the Laboratory of Genetics of TEST Lab for 2 weeks were trypsinized and expanded.

### **2.2 Adult human tissue isolation and cell culture**

Human AFSC were isolated as previously described.[16] Human amniocentesis cultures were harvested by trypsinization and subjected to c-Kit immunoselection by MACS technology (Miltenyi Biotec, Germany). AFSC

were subcultured routinely at 1:3 dilution and not allowed to expand beyond the 70% of confluence. AFSC were grown in culture medium ( $\alpha$ MEM) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from EuroClone Spa, Milano, Italy).

### 2.3 Exosome isolation from conditioned medium

Human AFSC isolated from four amniotic fluid cells were grown in 75 cm<sup>2</sup> flask until subconfluence (around  $1 \times 10^6$  cells). Before exosome extraction, the cells were maintained for 4 days in 10 ml culture medium deprived of FBS in order to exclude the contamination by exosomes comprised into FBS solution. The secreted part of the conditioned medium (CM) was then concentrated up to 2 ml using Centrifugal Filter Units with 3 K cutoff.[12] Then, the concentrated CM was treated with total exosome isolation solution from cell culture media (Invitrogen, Life Technologies, Carlsbad, California), according to manufacturer's instructions. The pellet (Exo) was collected and quantified by Bradford method. To obtain a sample for Western blot analysis, the pellet was re-suspended in lysis buffer. For in vitro and in vivo experiments, exosomes were re-suspended in PBS.

### 2.4 THP-1 cell culture

Human monocytic THP-1 cells (ATCC, Rockville, Maryland) were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, California) supplemented with 10% FBS, 2 mM L-Glutamine and 1% antibiotics (penicillin and streptomycin) (Sigma-Aldrich, St. Louis, Missouri). Differentiation of THP-1 cells into M0, M1, and M2 macrophages was performed as described by Genin in 2015.[17] Briefly, THP-1 cells were differentiated into M0 macrophages by incubation with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) for 6 hr (Sigma-Aldrich). Once the cells were adherent, were then polarized to M1 macrophages by incubation with LPS (100 ng/ml) and IFN- $\gamma$  (20 ng/ml) for 48 hr and M2 macrophages by incubation with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) (all from Peprotech, London, UK) for 72 hr.

During cell polarization protocols, M0, M1, and M2 THP-1 ( $5 \times 10^6$  cells) were exposed to exosomes (30  $\mu$ g of proteins/ $1 \times 10^6$  cells).

### 2.5 Preparation of protein extracts and western blot analysis

Cell extracts were obtained as described by Maraldi et al.[18] Briefly, subconfluent cells were extracted by addition of AT lysis buffer (20 mM Tris-Cl, pH 7.0, 1% Nonidet P-40; 150 mM NaCl; 10% glycerol; 10 mM EDTA; 20 mM NaF; 5 mM sodium pyrophosphate; and 1 mM Na<sub>3</sub>VO<sub>4</sub>) and freshly added Sigma Aldrich Protease Inhibitor Cocktail at 4°C for 30 min. Cell or exosome lysates and a small volume of the concentrated CM were sonicated, cleared by centrifugation and boiled in SDS sample buffer, then centrifuged. Supernatants were loaded onto SDS-polyacrylamide gel, blotted on Immobilon-P membranes (Millipore, Waltham, Massachusetts). The membranes were horizontally cut depending on the molecular weight of the protein of interest, then processed by western blot with the indicated antibodies, and detected by Supersignal substrate chemiluminescence detection kit (Pierce, Rockford, Illinois). Membranes were subjected to the stripping process (NaOH 0.2 N) in order to examine other proteins with similar molecular weight. Quantitation of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440CF and analysis with the Kodak 1D Image software. Primary antibodies were raised against the following molecules: HGF, Arginase 1, IDO and  $\beta$ -actin (Santa Cruz, California), TGF $\beta$ , CD86 (Novus Biologicals, Milano, Italy), RAB5 (HansaBioMed, Tallinn, Estonia), CD63, CD81 (Thermo Fisher, California), iNOS (Chemicon, California) and CD9 (Invitrogen).

### 2.6 Surgery and transplantation procedure

For implantation, in the study 20 CD® rats 8 weeks old (Charles River Laboratories, Lecco, Italy) were used as reported here below and in Figure S1. To evaluate the cartilage damage in different conditions, the animals were divided into three groups:

- Group 1 (four animals): OA joint, treated with 2 mg/50 µl/knee MIA intra-articular injection (MIA, Sigma Aldrich, Milano), a concentration comparable with the literature.[19, 20]
- Group 2 (eight animals): OA joint + CELLS, treated with  $5 \times 10^5$  AFSC 3 weeks after MIA.
- Group 3 (eight animals): OA joint + EXO, treated twice (10 days of distance) with 100 µg exosomes 3 weeks after MIA.

Animals were anesthetized with ketamine (Ketavet 100, Intervet Productions Srl, Aprilia, Italy) 70 mg/kg and xylazine (Sedaxylan, Eurovet Animals Health B.V., Bladel, Netherlands) 5 mg/kg. MIA was dissolved in physiological solution and the solution was injected by a 26 gauge syringe in the right knee.

AFSC were resuspended in 5 mM glucose/PBS and diluted into a concentration of 10,000 cells/µl. Under anesthesia, animals received a unilateral injection of cells (total 500,000 cells in 50 µL) and exosomes (100 µg in 50 µL) into the knee pretreated with MIA. This number of cells was chosen on the basis of the observation that 100 µg of exosomes, the same quantity used by Zhang et al,[21] are produced by 500,000 of AFSC cells. Four rats were injected only with 50 µL of glucose/PBS, as controls. By using a 26-gauge syringe, cells were introduced slowly (at a rate of 50 µL/min) into a joint. The needle was left in place for 2 min and then withdrawn slowly. After surgery, animals were placed individually into cages and allowed to survive for 3 weeks. Animals were then euthanized CO<sub>2</sub> inhalation.

All experiments were carried out according to the Bioethical Committee of the Italian National Institute of Health approved in the protocol n° 347/2015-PR. Animal care, maintenance, and surgery were conducted in accordance with Italian Law (D.Lgs 26/2014) and European legislation (2010/63/UE).

## 2.7 PAM–knee joint withdrawal threshold measurement

The Pressure Application Measurement (PAM from Ugo Basile, Italy) consists of a force transducer mounted on a unit fitted to the operator's thumb. The thumb unit is connected to a recording base unit containing the control panel and digital readout display. A gradually increasing squeeze force was applied across the joint at a rate of approximately 120 g/s ensuring the maximum test duration was 15 s. By means of calibrated instrumentation, the force in grams applied was displayed on the digital screen and recorded. The test endpoint was when the animal withdrew its limb or showed any behavioral signs of discomfort or distress. The peak gram force (gf) applied immediately prior to limb withdrawal was recorded by the base unit, and this value was designated the limb withdrawal threshold (LWT). Five measurements of both the ipsilateral and contralateral limbs were made at 1 min intervals during which the animals were returned to their respective cages. The mean LWTs were calculated.[22]

## 2.8 Histology

The knee joints were postfixed for 2 hr with 4% paraformaldehyde (pH 7.4) then rinsed in PBS. Contralateral joints were fixed as healthy negative controls. The fixed samples were treated with Surgipath Decalcifier I, Leica, for 3 weeks until complete decalcification. Samples were dehydrated with graded ethanol, diaphanized and embedded in paraffin. Sagittal plane serial sections (5 µm thick) were cut through the whole joint. Routine hematoxylin/eosin (H&E) and safranin-O/fast green (Saf-O) staining were performed in order to analyze

morphological details. Briefly, sagittal sections obtained every 50  $\mu\text{m}$  across the medial femorotibial joint were used to determine the maximal scores. The percentage of the exposed bone, cartilage and fibrous tissue in the different groups after 3 weeks of treatment was evaluated with ImageJ software. The quality of cartilage repair was assessed using the OARSI histologic grading system by three blinded independent observers for parameters including cellular morphology, matrix staining, surface regularity, structural integrity, thickness, bonding, and freedom from cellular changes of degeneration with a maximum grade of 6.[23]

Immunohistochemistry was performed using mouse anti-human mitochondrial protein (Millipore), anti-Sox9 (Cell Signalling), anti-collagen II (Calbiochem, Darmstadt, Germany), anti-iNOS (Chemicon), and anti-arginase 1 (Santa Cruz).[24] In brief, endogenous peroxidase activity was quenched by first incubating sections in 10% methanol, and 3%  $\text{H}_2\text{O}_2$ , followed by three washes in Tris-buffered saline (TBS, pH 7.4), and incubation for 1 hr in 3% BSA in TBS with 0.2% Triton-X-100 (TxTBS). Sections were incubated overnight in the primary antibody in TxTBS. Following  $3 \times 10$  min washes, tissue sections were incubated for 1 hr in peroxidase-labeled anti-mouse (Amersham, UK). Following another three washes, staining was visualized using diaminobenzidine (2 mg/ml) and  $\text{H}_2\text{O}_2$  (0.3  $\mu\text{l}/\text{ml}$ ) (all from Sigma-Aldrich). Sections were then stained with Harris hematoxylin. Images of histological samples were obtained by a Zeiss Axiophot microscope equipped with polarizer filters and with a Nikon DS-5Mc CCD color camera.

## 2.9 Statistical analysis

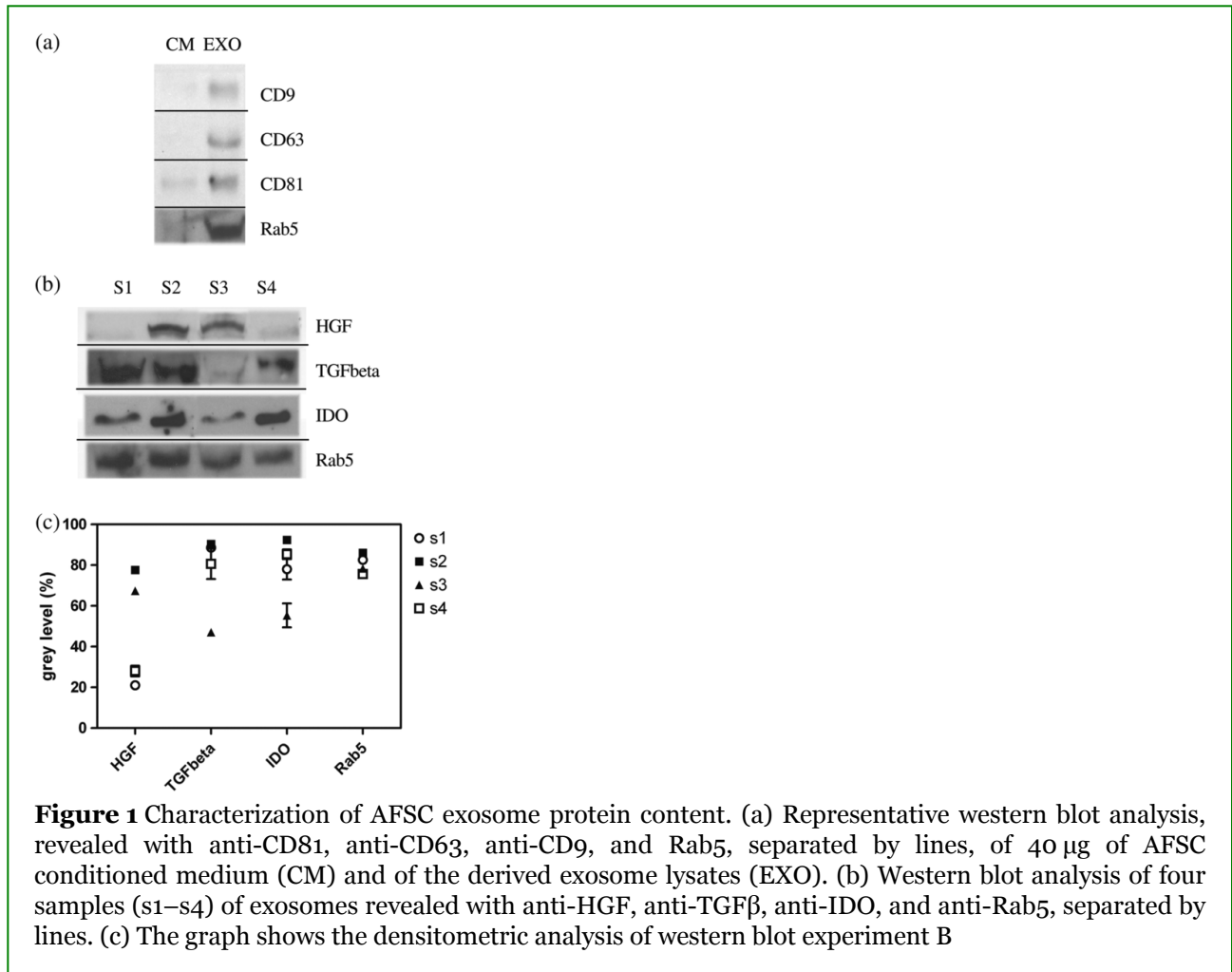
In vitro experiments were performed in triplicate. For quantitative comparisons, values were reported as mean  $\pm$  SE based on triplicate analysis for each sample. To test the significance of observed differences among the study groups Student's *t* test or one-way ANOVA with Bonferroni post hoc test were applied. A *p* value  $< .05$  was considered to be statistically significant. Statistical analysis and plot layout were obtained by using GraphPad Prism® release 5.0 software.

## 3 RESULTS

### 3.1 Characterization of AFSC' exosomes

A commercial kit was used for exosomes isolation, providing vesicles with dimensions compatible with one of the exosomes, as we previously reported.[12] The secreted vesicles purified from all four AFSC-CM displayed the accepted exosome associated markers, CD9, CD63, CD81, and Rab5,[25] as shown by western blotting in Figure 1a. The exosome concentration protocol was efficient, since these markers were easily detectable in the EXO sample, while in concentrated CM sample only CD81 and Rab5 were visible.





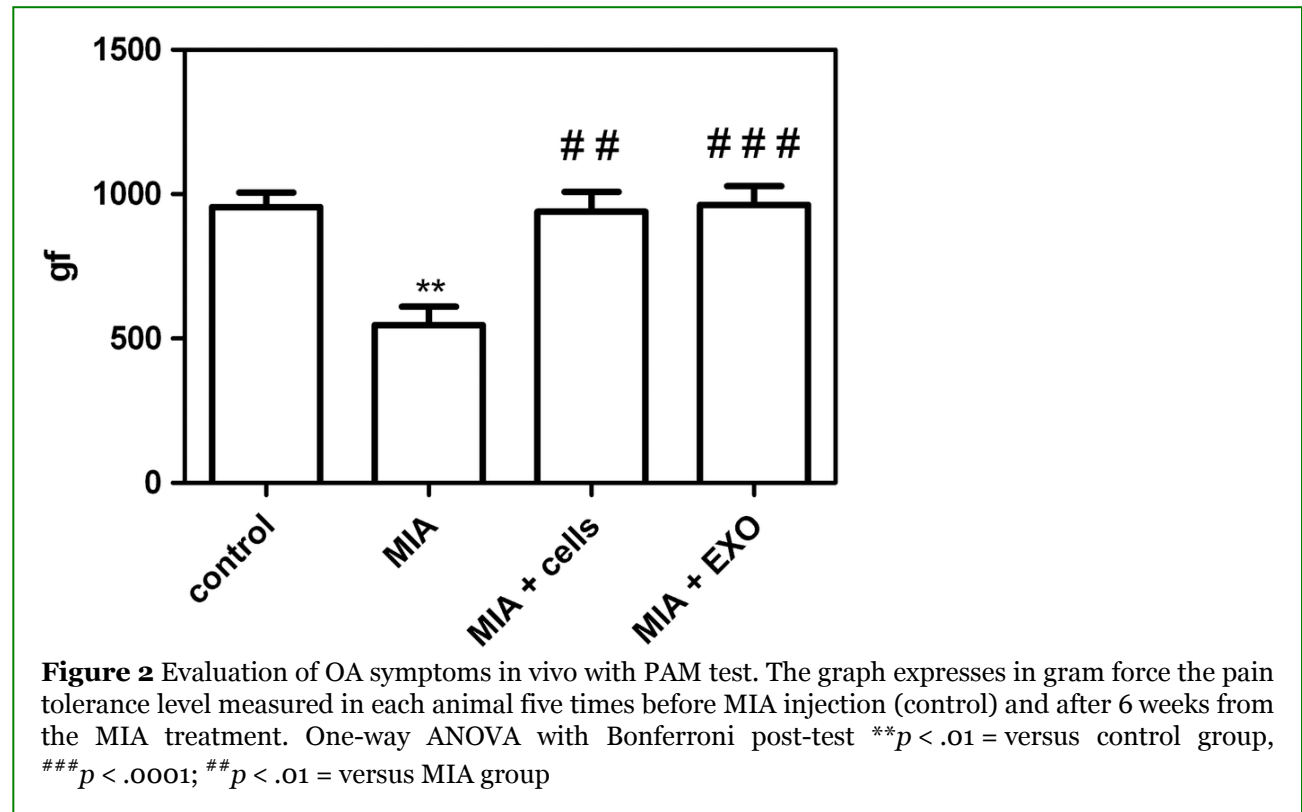
**Figure 1** Characterization of AFSC exosome protein content. (a) Representative western blot analysis, revealed with anti-CD81, anti-CD63, anti-CD9, and Rab5, separated by lines, of 40  $\mu\text{g}$  of AFSC conditioned medium (CM) and of the derived exosome lysates (EXO). (b) Western blot analysis of four samples (s1–s4) of exosomes revealed with anti-HGF, anti-TGF $\beta$ , anti-IDO, and anti-Rab5, separated by lines. (c) The graph shows the densitometric analysis of western blot experiment B

Then we checked the expression of proteins with immunomodulatory role, such as HGF, TGF $\beta$ , and IDO, in compliance with our previous data.[12] Figure 1b shows that the presence of these factors in exosome samples, derived from four AFSC, is donor dependent. Indeed, HGF is the most variable protein, compared to IDO and TGF  $\beta$ . Rab5, as exosome marker, was used as loading control beside the obvious same protein amount per lane, so its presence is almost equal, as shown in the densitometry graph (Figure 1c).

### 3.2 Effect of human AFS cells and exosomes on the repair of cartilage defects

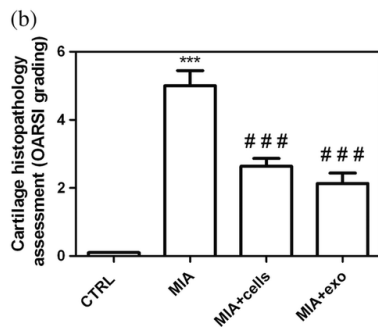
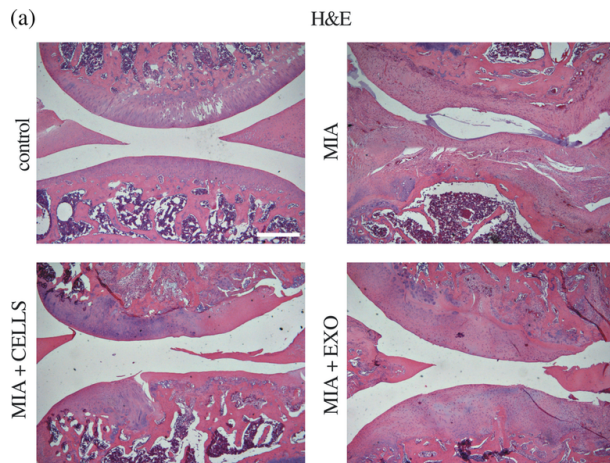
We induced osteochondral defects in a rat model, by an intra-articular injection of MIA, as described in M&M section. Briefly, in each animal, PAM test was performed prior to MIA injection and then once a week, in order to follow the progression of OA induction (Figure S1). Preliminary experiments defined 3 weeks as the necessary time for obtaining a significantly different pain level measure between the healthy and MIA groups. This timing is consistent with data reported in literature[26] and it was confirmed also in the experiment shown in this paper. Then, animals were treated with vehicle (PBS), with AFSC ( $5 \times 10^5$ /defect) or with 100  $\mu\text{g}$  exosomes, this repeated after 10 days. The weekly measure by PAM test showed that, after only 3 weeks, the pain threshold of CELLS- or EXO-treated OA-animals was comparable to the healthy group, while it has been halved in OA group (Figure 2). Measure performed before the second exosome injection at day 7 showed that the effect of CELLS and EXO is already visible but not yet significant (data not shown). Looking singularly at each AFSC sample and the derived-exosomes, all the cell-treated animals showed pain threshold similar to

the healthy group, while, in only one case, exosome-treatment (sample 3) seemed not to be effective (data not included).



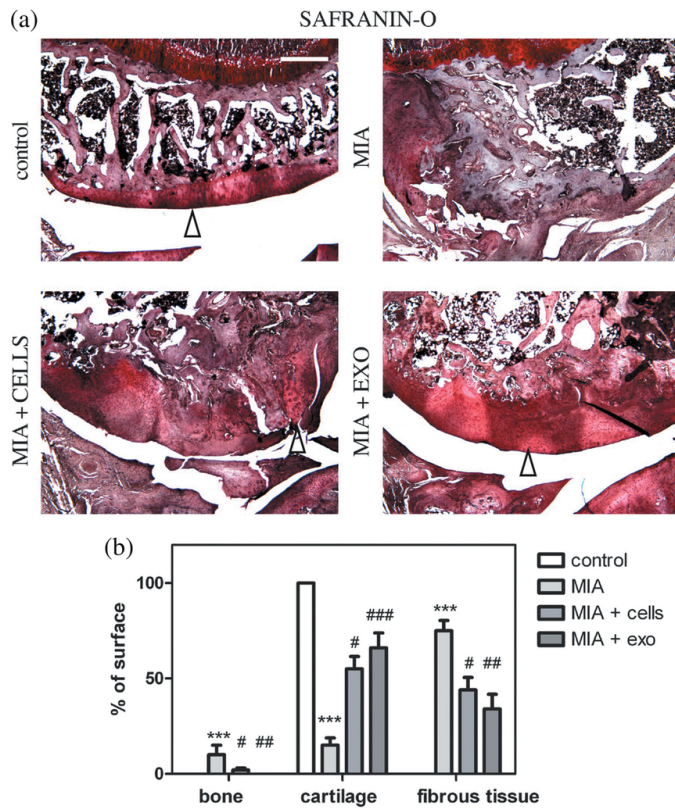
Basing on the symptom point of view, we decided to stop the in vivo experiment for all the animals, so the whole joints were harvested for histological analysis.

Figure 3a shows representative images of H&E staining of negative control, positive control (MIA) and treated samples with CELLS or EXO. Histologically MIA treatment at 6 weeks displays huge damage on the joint surface and also in the subchondral bone, as expected. Moreover, the joint area is filled with connective tissue. Most of exosome-treated defects showed complete neo tissue filling with good surface regularity, while cell-treated defects had a few fissures on the surface. The evaluation with OARSI grading (Figure 3b) clearly indicated that both the treatments significantly improved the cartilage features, moving from grade  $5 \pm 0.4$  for MIA to  $2.5 \pm 0.2$  and  $2 \pm 0.3$  for CELLS and EXO, respectively, justifying the behavioral results obtained with PAM test. For EXO sample 3, the negative result was confirmed (data not shown).



**Figure 3** Histological evaluation of OA signs. (a) Representative images of H&E staining of negative control, MIA alone or treated for 3 weeks with AFSC (MIA + CELLS) or exosomes (MIA + EXO). Scale bar = 500  $\mu$ M. (b) Graph of OARSI grading that defines the severity level of OA. One-way ANOVA with Bonferroni post-test. \*\*\* $p < .0001$  = versus control group, ### $p < .0001$  = versus MIA group

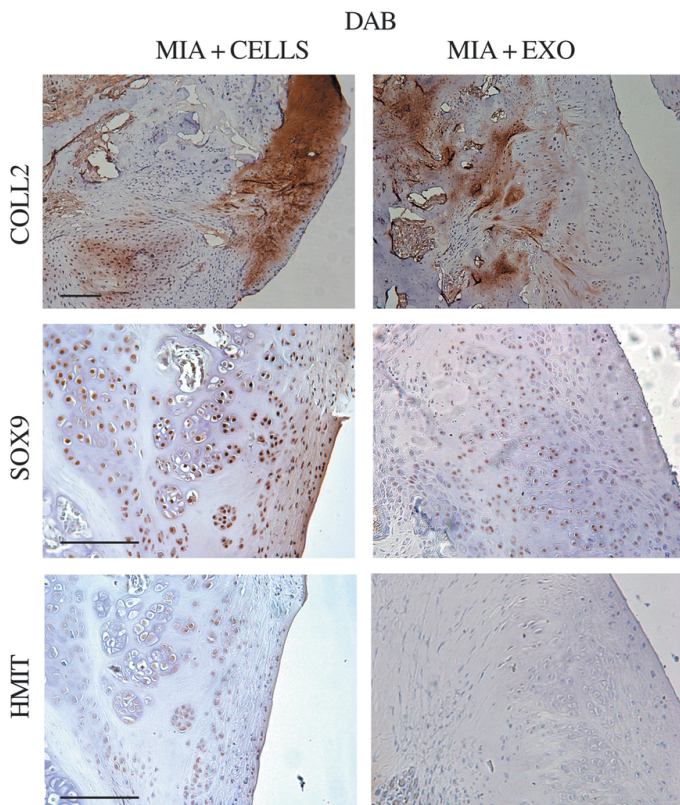
The characterization of matrix components was then approached initially by safranin-O staining, that colors in red amounts of glycosaminoglycan (GAG): Figure 4a points out that in MIA samples the extra-cellular matrix is intensely red stained only in the epiphyseal growth plate, while the articular surfaces are pale and bone tissue is exposed. Into CELLS samples safranin stains part of the superficial tissue, on the contrary in the EXO ones GAG distribution appeared uniform.



**Figure 4** Characterization of joint tissue composition. (a) Representative images of safranin-O staining (in red indicated by arrow heads) of negative control, MIA alone or treated for 3 weeks with AFSC (MIA + CELLS) or exosomes (MIA + EXO). Scale bar = 500  $\mu$ M. (b) Graph of quantitative tissue analysis measuring the percentage of tissues, such as bone, cartilage, fibrous tissue, covering the joint surface. One-way ANOVA with Bonferroni post-test. \*\*\* $p < .0001$  = versus control group, ### $p < .0001$ ; ## $p < .01$ ; # $p < .05$  = versus MIA group

Analysis of tissue features is collected in Figure 4b and quantitatively supports these observations: the exposed bone tissue, present in MIA group, is covered in all EXO and CELLS samples, meanwhile fibrous-connective tissue and cartilage are both at 50% of the CELLS sample surfaces. Therefore the most promising results are the one with EXO, where cartilage tissue significantly overbears fibrous tissue.

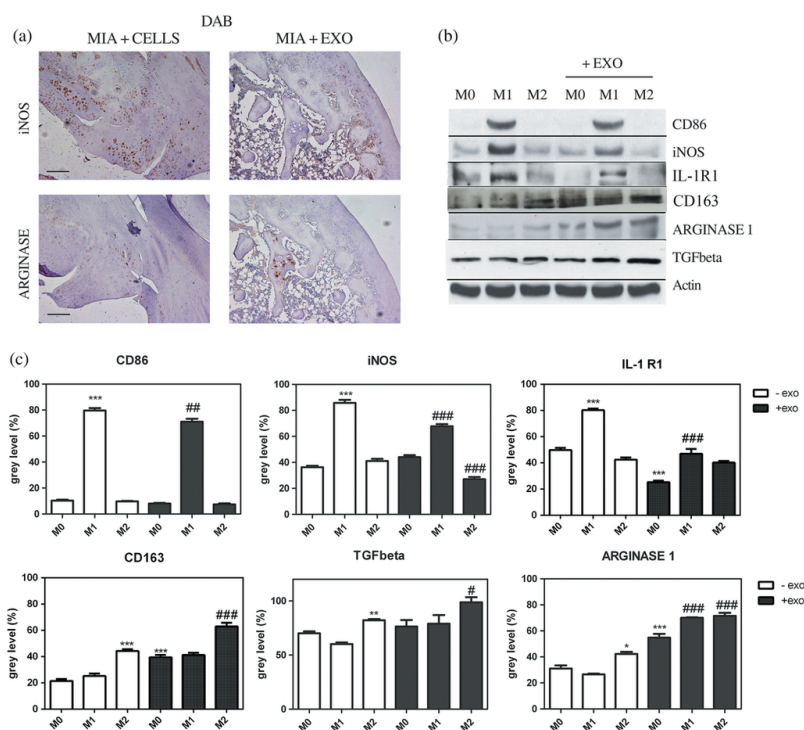
The presence into the neo tissue of typical markers of hyaline cartilage was revealed with immunohistochemistry by using antibodies directed to collagen II, the more abundant matrix fiber, and Sox9, a transcriptional factor required for cartilage formation. Figure S2 reports <<Query: Please provide a file and a suitable caption for Figures S2 and S3. Ans: I send the files by @mail, as suggested.>> representative images of control and MIA samples stained for collagen II and Sox9. Figure 5 shows that collagen II staining is more intense in the CELLS sample, although with a minor spread distribution, if compared to EXO sample. This regular distribution of fibers is consistent with the one of chondrocytes (Sox9 positive cells) in the last, meanwhile they are enclosed in groups in CELLS sample. These are the same cells positive for the antibody that recognizes only human mitochondrial protein (HMIT).



**Figure 5** Evaluation of hyaline cartilage markers. Representative images of rat joints treated with AFSC (MIA + CELLS) or exosomes (MIA + EXO) stained with DAB after labeling with anti-collagen II, anti-Sox9 or anti-human mitochondria protein (HMIT). Scale bar = 500  $\mu$ M

### 3.3 Exosome role on macrophage polarization

Since a lymphocyte infiltration was not observed into the treated joints, we investigated the presence of macrophages, by antibodies directed to iNOS and arginase 1, characteristic enzymes of M1 and M2 macrophages, respectively. Figure S3 reports representative images of control and MIA samples stained for iNOS and arginase 1. Figure 6a shows positive cells in both CELLS and EXO joints, but a defined arginase staining prevails in the second one.



**Figure 6** Macrophage polarization: role of AFSC-derived exosomes. (a) Representative images of rat joints treated with AFSC (MIA + CELLS) or exosomes (MIA + EXO) stained with DAB after labeling with anti-iNOS and anti-arginase 1. Scale bar = 500  $\mu$ M. (b) THP-1 monocyte cells induced to macrophage (Mo) were treated to polarization protocol (see M&M) in the presence or absence of 80  $\mu$ g of exosomes for 72 h. Immunoblot analysis was performed with anti-CD86, anti-iNOS, anti-IL-1 R1, anti-CD163, anti-arginase 1 and anti-TGF $\beta$ , separated by lines. Actin was revealed as the loading control. (c) The graphs show densitometric analysis of western blot experiments B. One-way ANOVA with Bonferroni post-test. \*\*\* $p < .0001$ ; \* $p < .05$  = versus M0 untreated with exosomes, ### $p < .0001$ ; ## $p < .01$  = versus the corresponding sample untreated with exosomes. Student's  $t$  test \*\* $p < .01$  versus M0 untreated with exosomes; # $p < .05$  versus the corresponding sample untreated with exosomes

In order to evaluate the role of exosomes in the macrophage activity, we performed an in vitro test by using a polarization protocol of the THP-1 monocyte cell line. So, monocytes were induced to macrophage by PMA, then, during incubation with polarization media toward M1 and M2 subpopulations, cells were exposed to exosomes. Western blot analysis (Figure 6b) on not treated cells revealed the higher expression of CD86, iNOS, and IL-1 R1 in M1 sample, since they are typical markers of inflammatory macrophages, meanwhile CD163, arginase 1, and TGF $\beta$ , markers of resolving macrophages, were more intense in M2 cells, confirming that the polarization protocol worked properly. Densitometric analysis (Figure 6c) highlighted that exosome co-treatment limited M1 polarization, indeed CD86, iNOS, and IL-1 R1 expression significantly decreased. In parallel, the only exposure to exosomes improved the production also in M0 cells of molecules involved in the anti-inflammatory role of macrophages, such as CD163, arginase 1, and TGF $\beta$ .

## 4 DISCUSSION

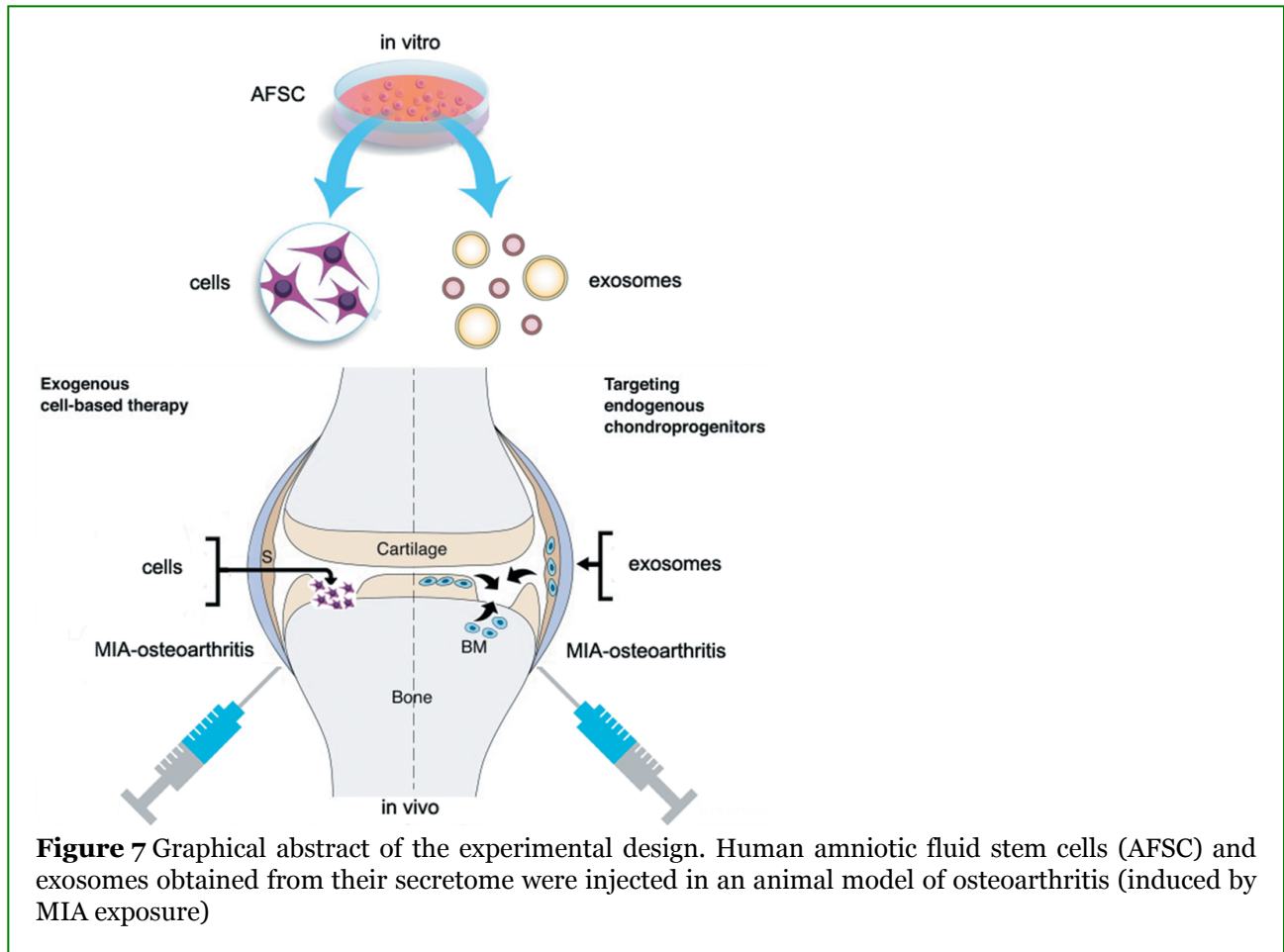
OA is a degenerative pathology of the whole mobile joint where cartilage and bone are the main targeted tissues during the disease course, which is based on the occurrence of a condition of chronic inflammation that involves a drastic decline in the quality of life. Although the use of MSCs for cartilage repair is motivated by their potential to differentiate into chondrocytes to replace dead or degenerated chondrocytes, it is increasingly evident that the repair potential of MSCs derives from its secretion.[27, 28] Among the trophic factors present

in the secretome, exosomes play an important role in the mediation of the paracrine effects of MSC.[29, 30] By comparing them with cell therapies, MSC-derived exosomes would offer the advantage of being cell-free, ready for use and more susceptible to reformulation to support different routes of administration. However, deeper studies are needed to identify the components present in MSC exosomes and possibly involved in the mechanisms underlying cartilage repair. It has recently been demonstrated that exosomes obtained from MSC of embryonic origin initiate a rapid repair and regeneration of osteochondral defects through a multifaceted mechanism that coordinates the modulation of different cellular processes such as migration, proliferation, matrix synthesis, macrophage infiltration and cytokine production for optimal tissue repair.[21, 31] As just mentioned, these studies show the efficacy and the mechanism of action of exosomes from embryonic stem cells; the obtained indications are certainly of great interest, but applicability remains an obstacle, lives the underlying ethical problems. We study stem cells of human amniotic fluid, which is a source of easy availability and free of ethical implications, but from which stem cells are widely multipotent, because of early fetal origin, and easily expandable in culture, that have proved effective in bone regeneration.[13, 14] However, donor-dependent differences among AFSC have been reported.[16] Comparing with adult stem cells, such as bone marrow, amniotic fluid MSCs are comparable sources of exosomes in size distribution, morphology, and expression of typical surface markers, but yield may be higher from amniotic fluid cells. Therefore, amniotic fluid appears to be a preferable source of exosomes for clinical applications.[32]

Previously mentioned studies on the effect of exosomes on OA have been conducted using a surgical method models that may be too quick in order to follow the early stages in OA development as well as for measuring early drug treatment.[15] In this study, we have therefore decided to use an induction method of OA that reflects some typical aspects occurring in aging, rather than following trauma, that is, an intra-articular injection of MIA that causes already after 3 weeks a diffuse degeneration of the joint surfaces, as shown by the histological images. By inducing OA with a simple injection, the damage affects the components of the intra-articular surfaces, without involvement of adjacent tissues that are damaged by collagenase OA induction: indeed, intra-articular administration of collagenase breaks down type I collagen within the cartilage leading to decreased collagen matrix in the tendons and ligaments, consequently leading to joint instability.[15]

The behavioral analysis PAM test that measures the pain threshold endured by the animal, allowed us to monitor, during the weeks, the effectiveness of the treatments, that is, the same induction of OA with MIA, or the resolution of damage thanks to exosomes (EXO) or stem cells from amniotic fluid (CELLS). Thanks to these tests we could define during the course of the weeks, when it was more appropriate to stop the experimentation. We have in fact sacrificed the animals after only 3 weeks, that is when the PAM test showed us that the rats with OA treated with EXO or CELLS had regained a pain tolerance similar to that of the healthy animal, suggesting that the damage was being resolved. In this way, we were able to grasp the optimal time for the subsequent evaluation of the histological aspects.

Another limit shown by the literature on the use of exosomes is that the therapies require repeated infusions of exosomes (one per week), while the therapies with cells are conducted with a single injection. In our experimental design (Figure S1 and Figure 7) we wanted to compare, for the first time, the efficacy of the exosomes with that of the AFSC from which they were obtained. For this reason, we infused a number of cells, half a million per articulation, obtainable with a short expansion time of cells in vitro up to fifth passage, and in line with a recently published study,[33] even if in the literature are mostly used 1 million of cells. The medium, enriched by the secretoma produced by 1 million AFSC for 4 days in culture, was collected and the content in exosomes was isolated. These exosomes were then injected into the joint damaged by MIA in just two tranches, spaced by 10 days. In this way the two treatments seem comparable, as it is true that the implanted cells remain to secrete their secretoma for 3 weeks, while the injected exosomes are the result of the secretion of 8 days (4 plus 4), but we must consider that some collected cells can easily be damaged during the transport and injection process, and that, once injected, can be partly eliminated from the animal's immune system. We have in fact used immunocompetent animals in order to follow the physiological anti-inflammatory response, aware of the fact that MSCs, such as AFSC, cause a poor rejection, lacking some histocompatibility factors (MHC I).[34]



The histological analyses have confirmed what was anticipated by behavioral tests: we have in fact observed that exosomes and AFSC improve tissue damage induced by MIA. In fact, we observed the presence of newly formed cartilaginous tissue covering the articular surface, while the fibrous tissue is much less expressed than the samples in which the regeneration was autonomous, that is, only with MIA. In some points of the newly formed tissue of the samples treated with AFSC, some human cells are present, as shown by immunohistochemical analyses, which demonstrate their engraftment and differentiation, involvement, without lymphocytes infiltration, at least at 3 weeks. Comparing the results obtained with AFSC and exosomes, we can see how the difference in behavioral aspects is slight, while the effectiveness of some exosome sample in the repair of cartilage tissue is more evident, since it is much more regular, continuous and mature. There is indeed a difference among the samples of exosomes that emerges even when the content of immunomodulatory proteins is analyzed. From these preliminary data on a pool of exosomes taken from four individuals, we observed that there could be a positive correlation between the content in the exosomes of TGF $\beta$  and IDO, rather than of HGF, and their effect of the resolution of damage from OA. In fact, these molecules are known as immunosuppressive, as they stimulate the Treg lymphocytes and instead inhibit the Th, NK lymphocytes, and the maturation of inflammatory macrophages.[35] Furthermore TGF $\beta$  is also a factor that stimulates chondrogenesis, as it promotes the expression of Sox9 and collagen II.[36] The observation that one sample of exosome did not exert an efficient regenerative effect unlike its AFSC source could be explained by the fact that exosomes were collected from CM of AFSC cultured in vitro, without any stimulus, while injected AFSC have been exposed to OA environment, possibly activating a secretome production more specific for the inflammatory status. A further study focused on the different efficacy of exosomes derived from a larger cohort of donors should be conducted.

We previously demonstrated that AFSC exosomes have a negative effect on in vitro lymphocyte proliferation,[12] but by observing the histological specimens of the MIA-induced OA joints we did not observe



lymphocyte. We then investigated the presence of markers for inflammatory or anti-inflammatory macrophage cells (M1 and M2, respectively). Indeed, it is known that M1 polarized macrophages in synovial tissues of OA inhibit the chondrogenic differentiation of MSC while M2 supports the survival of cartilage graft.[37] Immunohistochemical data show that treatment with exosomes promotes the involvement of M2, compared to AFSC-treated, thus inducing subsequent faster cartilage repair.

To demonstrate this effect of AFSC exosomes, we tested the in vitro polarization modulation of macrophages in the presence of exosomes. We used the THP-1 monocyte line induced in the two polarization pathways and we monitored the expression variation of typical markers of M1 and M2 in the presence or absence of exosomes. The data obtained confirm that the content of the exosomes, which certainly includes a multitude of protein and nucleic acid factors, is able to regulate the macrophage population, promoting the anti-inflammatory component, that is, M2, supporting the data we observed in vivo. It has been demonstrated that TGF- $\beta$  itself induces a M2-like phenotype characterized by up-regulation of the anti-inflammatory cytokine IL-10, and down-regulation of the pro-inflammatory cytokines TNF- $\alpha$  and IL-12.[38] This is consistent with the immunomodulatory properties previously reported in MSC exosomes: MSC exosomes could induce the expression of anti-inflammatory molecules, IL-10 and, with a positive loop, TGF- $\beta$ 1, and attenuate the expression of pro-inflammatory, IL-1b, IL-6, TNF- $\alpha$ , and IL-12P40, in monocytes THP-1.[30]

## 5 CONCLUSION

Here have shown that exosomes derived from AFSC generate an advanced cartilage repair by inducing the migration and proliferation of appropriate types of repairing cells and promoting cartilage matrix synthesis. Our study demonstrates for the first time that intra-articular injection of human AFSC exosomes counteracts degeneration of cartilage across the surface in an adult immunocompetent rat model. In this study, no adverse inflammatory response was observed in all animals, even those treated with human cells, supporting the potential application of exosomes and human AFSC cells in allogeneic human recipients.

However, the mechanistic role of AFSC exosomes in modulating the immune microenvironment to a regenerative and less pro-inflammatory phenotype during cartilage repair should be further investigated. For example, it would be crucial to determine in future studies whether exosomes induce greater migration or polarization of M2 macrophages during cartilage repair. Furthermore, this is a proof-of-concept study performed in the model of OA in the rat. Further investigation into a larger animal model, such as rabbit or pig, would be necessary. Collectively, this study demonstrates that it is at least equivalent to use a “cell-free” strategy instead of using human AFSC for cartilage repair, far exceeding the impediment and limitations of current cell therapies.

## CONFLICT OF INTEREST

The authors report no conflict of interest. We declare that there was not a role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

## AUTHOR CONTRIBUTIONS

M.Z. performed in vivo experiments; F.B. performed histological analysis; F.C. performed exosome extractions; E.B. performed amniotic fluids and informed consent collection; T.M. is responsible for the design of the work, acquisition, and interpretation of data and drafting the manuscript. All authors read and approved the final manuscript.

## ETHICS STATEMENT

An informed consent allowing the use of clinical data and biological samples for the specified research purpose (protocol 360/2017 dated December 15, 2017) was signed by all infertile couples before treatment and collected by the Unit of Obstetrics & Gynaecology, Policlinico of Modena (Italy).

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