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Development of Human Chondrocyte–Based Medicinal Products for Autologous Cell Therapy

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1. Introduction

A cell therapy is a clinical treatment including an *ex vivo* cell manipulation step. Such a therapeutical option began more than forty years ago and is now a worldwide reality. Many human cell-based clinical trials have been developed in every medicine's field mostly to cure diseases where conventional treatments are inadequate. Even though there have been few completed trials and some conflicting results on their effectiveness have been reported, the full potential of cell-based treatments remains to be explored and investigated (Park et al., 2008). Moreover, public expectation for such novel therapies, especially for treating incurable and/or rare diseases, remains high. Nowadays each tissue of the human body, including foetal and embryonic ones, can become a reliable source for cell therapy (Mason & Dunnill, 2009). Cells isolated from a specific source can be used also to cure every other tissue of the body and may be administered alone, in combination with biomaterials, scaffolds, cytokines and growth factors or can be genetically manipulated (gene therapy). Cell administrations can be local or systemic, singles or multiples. Treatments may be autologous or allogeneic (from living or cadaver donors). A cell preparation can be crucial for a treatment such as in bone marrow transplantation or otherwise it may be used as an adjuvant to improve clinical results like in regenerative medicine or to slow down the development of several chronic conditions. Cell effect after treatment can be via the ability to differentiate along several lineages or, as recently highlighted for stem cells, also via the capacity to release anti-inflammatory cytokines, growth factors and proteins, collectively known as paracrine factors, which may modulate the host microenvironment by stimulating endogenous stem cells recruitment, differentiation and angiogenesis, thus acting as real drugs (Yagi et al., 2010). *Ex vivo* cell manipulation protocols are different, depending on cell source, type, target, disease and Country regulations. Current European cell therapy laws classify manipulation types according to potentially associated risks. Cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, lyophilization, freezing, cryopreservation and vitrification are considered "minimal manipulations". On the other

hand, cell processing like induction to proliferation, non-homologous use (if cells or tissues are not intended to be used for the same essential functions in the recipient as in the donor) and association with scaffolds or medical devices are defined as "extensive" or " substantial" manipulations. These new kind of extensively manipulated cell-based products are termed "medicinal product for advanced cell therapy" (see Regulation (European Commission [EC]) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004). The term "medicinal" is not only a definition, but, of course, holds specific technical and practical consequences entering these products in the "drug world". In fact, pre-clinical and clinical data that are necessary to demonstrate their quality, safety and efficacy should be highly specific. Moreover, it is also mandatory that they have to be manufactured like medicinal products. To this end, it is primarily required to have a suitable environment built as a real pharmaceutical factory and working according to specific rules named Good Manufacturing Practices (GMPs). GMPs are worldwide guidelines for the management of manufacturing and quality control of pharmaceutical products. The Food and Drug Administration [FDA] enforces GMPs in the United States while the European Medicines Agency [EMA] in Europe (The Rules Governing Medicinal Products in the European Union, Volume 4-Guidelines for Good Manufacturing Practices for medicinal products for human and veterinary use, current Edition) where manufacturing must be authorised by the competent Agency of each Member State. Such a structure (also named "cell factory" or "cleanroom facility") allows minimization of any contamination risk by means of standardization and continuous monitoring of specific parameters such as air filtration and ventilation, temperature, relative humidity, differential pressure, number of air particles and bacterial colony forming units. Besides the environmental monitoring, cell culture and reagents must be checked for the presence of bacterial and viral contamination, mycoplasma and endotoxins. Furthermore, standard operative procedures, personnel training and process traceability should be developed and performed. Extensively manipulated cells are generally thought to be elaborate and costly, especially due to GMPs requirements. However they ensure three main characteristics: safety, product consistency and manufacturing quality. Considering that many cell therapies are still in an early experimental phase and that several aspects are not completely understood, these characteristics may represent a real guarantee for safe, standardized and controlled treatments. To date, proposed employments for cell-based medicinal products are quite impressive (Mason & Manzotti, 2010). Fields of interest are musculoskeletal tissue regeneration, autoimmune disorders, myocardial infarction, gastrointestinal diseases, urogenital system disorders, nervous system diseases, wound healing, plastic surgery, organ transplantation, graft versus host disease (GvHD) and diabetes (www.ClinicalTrials.gov). There is tremendous scope for applying cell therapeutics in the musculoskeletal system (Nöth et al., 2010). Cells can be used to repair or regenerate injured or diseased tissues (cartilage, bones, tendons, ligaments, muscles, etc..), or to treat chronic conditions such as Osteoarthritis or Rheumatoid Arthritis. As most cell therapy treatments for musculoskeletal diseases are not life-threatening, safety is a key issue for any clinical application.

1.1 Autologous chondrocyte implantation

Hyaline articular cartilage is a highly specialized tissue derived from mesenchyme during embryonic development. It has the main function to protect the joint by distributing loads

and thus preventing potentially damaging stress on the sub-chondral bone. At the same time it provides a low-friction bearing surface to enable free movements. Chondrocytes, the only cell type of mature cartilage, secrete and deposit around themselves a characteristics matrix composed primarily of water, collagens (mainly collagen type II), proteoglycans (mainly aggrecan) and other non-collagenous proteins (Becerra et al., 2010). Articular cartilage lesions are common in the general population and more often anticipated in young and physically active people. Despite the tissue is susceptible to damage, it has limited capacity for regeneration and repair because of poor vascular supply and lack of an undifferentiated cell population capable of migrating and responding to the insult (O'Driscoll, 1998). If left untreated, cartilage injuries may lead to pain and loss of function and predispose individuals to osteoarthritis in later life and eventually to requirements for total joint replacement (arthroplasty). The need of hospital attention is associated with a significant impact on quality of life and represents a huge socioeconomic burden to society. There is no uniform approach to managing cartilage defects (Harris et al., 2010). In younger, active patients "biologic" solutions that prevent or slow down tissue degradation process should be preferred. These procedures can be classified as palliative (arthroscopic debridement), reparative (microfracture) and regenerative such as pertiosteum, perichondrium or osteochondral grafting and Autologous Chondrocyte Implantation (ACI). Conventional treatments by debridement or microfracture produced various outcomes since the resulting repaired tissue is fibrocartilage which lacks the appropriate biochemical and biomechanical properties of normal healthy tissue. Currently, arthroscopic debridement and microfracture are commonly used as first-line treatment for symptomatic focal chondral defects that are relatively small with minimal associated bone loss. Regenerative procedures including ACI can be used as second-line measures to repair chondral or osteochondral defects. ACI therapeutical approach was first used by a Swedish Group (Brittberg et al., 1994) to treat full-thickness chondral defects of the knee and later applied to the ankle. The treatment is now suitable also for other joints such as hip. In the original procedure (first generation technique) small grafts of normal cartilage removed from non-weight bearing areas of the knee were treated in a proper laboratory. Individual chondrocytes were isolated, cultured in specific conditions, and, following a period of cellular division, retrieved for re-implantation. Cell suspension was then injected beneath a periosteal patch harvested from the proximal medial tibia and sutured to the defect. Clinical, radiological and histological results are available at 10 to 20 years after the implantation, suggesting that outcomes remain high, with relatively few complications (Peterson et al., 2010). First generation ACI revealed, however, several disadvantages, such as transplant hypertrophy, calcification, delamination, cell leakage in the articular environment and loss of phenotype due to previous monolayer expansion. Given these shortcomings, recent experimental and clinical research has been directed towards the development of second generation ACI procedures using suitable scaffolds which act as carriers for the implantable cells maintaining at the meantime phenotype stability (Iwasa et al., 2009). These engineered tissues are then cut to the correct size and shape of the defects directly in the operatory room. The scaffolds, which efficiently "mimic" the natural surroundings of cartilage cells, may have different origins (synthetic, natural), characteristics (fibers, gels, sponges, microspheres, etc.) and spatial organization (two- or three-dimensional). Third generation ACI uses three-dimensional (3D) matrices such as hyaluronic acid as scaffolds. The process of implantation in second and third generation techniques can also be performed arthroscopically or with a small incision. Recently, a new technique called 'Characterized

Chondrocyte Implantation' (CCI) utilizes a selected chondrocyte population that expresses a marker profile that can predict cell ability to produce *in vivo* hyaline-like cartilage (Saris et al., 2008). Many Authors in the Literature have suggested ACI effectiveness also for second and third generation techniques and these procedures are now widely diffused and utilized all over the world for cartilage defects repair. However, besides good outcomes evaluations, there is still scepticism about the use of ACI, particularly for its clinical and cost effectiveness in comparison with other traditional treatments and for the steeper learning curve, at least compared with marrow-stimulating techniques (Vasiliadis & Wasiak, 2010). Different Literature revisions highlighted that there is still insufficient evidence to draw conclusions and that further trials with long-term follow up are required in order to clarify ACI clinical benefits..

1.2 Cell manipulation in autologous chondrocyte implantation: from research to cleanroom

Cell manipulation for ACI is crucial step that have to be performed in compliance with GMPs in order to guarantee a safe, standardized and efficacious product to implant. In this perspective the development of a validated and a repeatable process becomes a key issue for this therapy. Chondrocytes can be easily isolated from articular cartilage tissue by enzymatic treatments and then cultured in different conditions like monolayer, bi/three-dimension, chemical or mechanical stimulation or inhibition. Several chondrocyte culture systems that have been developed display a huge number of applications in the research field as attested by worldwide publications. In fact they represent models for cartilage investigation, which is essential for identifying the pathways of both normal development and pathological degeneration and inflammation of the tissue (Roseti et al., 2007). Recently, there has been a great deal of interest in the *ex vivo* development of hyaline cartilage that can be utilized for the regeneration of damaged or diseased tissue. The realization that chondrocytes may act as drugs having therapeutical effects in cartilage regeneration led to new responsibilities and roles for cell culture's laboratory. ACI application required an integration work between clinicians and cell biology experts and it would not have been possible without the support of GMPs specialized laboratories or facilities. Obviously, clinical application of research models presents specific features of quality assurance which must be met. In particular, for cell cultures, a transfer technology step is required or, in other words, research protocols must be translated into GMPs' ones. This involves taking into account not only the peculiar nature of cells and culture's models, but also the mandatory compliance with current GMPs and all the specific cell therapy regulations. This chapter describes the transfer technology utilized to standardize the manufacturing of engineered chondrocyte-based products for applications in ACI. In particular, it focuses on the development and validation of a GMPs' compliant manufacturing process and then of "consistent" chondrocyte-based medicinal products. The GMP facility performing the below described validation processes is located in a public Hospital in Italy. Therefore specific Italian and European rules have been followed.

2. Development and validation of a GMPs compliant chondrocyte culture process suitable for clinical use

Process validation is a pre-requisite to ensure consistent manufacture. Cell processing such as in ACI is a long lasting, articulated process. It comprises three main manipulation phases

before final product packaging and release: cell isolation, expansion, and seeding onto biomaterials. For a complete GMPs compliance it is required that each step is validated singularly. One important thing to consider and to perform before starting is a careful evaluation and subsequent choice of high quality raw materials to be used in the process. Once the choice has been taken, the entire validation should be performed using the same materials. Changes are allowed, but a re-validation step is required.

2.1 The choice of raw materials

This is a highly critical step since raw materials, such as culture reagents or plastic wares, become directly in contact with the cells during the process. Moreover other materials, like scaffolds or cell supports, can become an integral part of the medicinal products. Therefore, the choice for such materials should be geared towards products ensuring the highest quality provided by the market at the moment. First of all, the cell producing facility should attest the quality assurance level of each Company/Institution that intends to choose as supplier. This investigation termed "supplier qualification" is mandatory for a fully GMPs compliance and should be performed not only by examining the accreditations provided by the Company itself but also by on-site inspections. Hence, material's full batch documentation/certification should be carefully evaluated to attest fulfilment with specific current regulations. For example, cell culture media sterility should be certified by specific analyses validated in compliance with current European Pharmacopoeia. In particular, it is required that these products are negative for aerobic, anaerobic bacteria, fungi and endotoxins. Documentation control is a delicate and important step, but not sufficient for entering materials into the process. In fact, each declared critical feature should be cross-checked by internal quality controls in the cell factory. Only after passing such internal controls, materials are considered adequate for the process and validation can start. An important point to consider for reagent choice is to avoid zoonosis risks. A recombinant origin should be preferred for enzymes such as Trypsin used for the rapid detachment of adherent cells, like chondrocytes, from the growth substrate and Collagenase type II utilized to digest cartilage and thus isolate cells. In this case users should control documentation also for recombinant source that must be clearly indicated. For animal origin products, like foetal bovine serum (FBS) as supplement in culture, high quality is mandatory. FBS should be free of microbial, mycoplasma and viral contaminations. More importantly, it must be stated and certified its origin from Bovine Spongiform Encephalopathy (BSE) free countries such as Australia and New Zealand. The country of processing should be indicated too, if different. The serum producing process should be described giving evidence to exclude any possibility from contamination with tissues that may harbour the BSE agent, such as the brain, spinal cord and distal ileum. If the final product encloses other components like scaffolds or biomaterials they should be appropriately characterised and evaluated for suitability for the intended use. Nowadays the market displays variability and availability of such materials. For application in cartilage regeneration they have to display several properties: biocompatibility, biodegradability and malleability to fit defects. Moreover they should be viscous and adhesive enough to allow chondrocyte trapping and fixation to the implantation site, respectively. Furthermore they have to guarantee cell viability, growth and phenotype stability (Iwasa et al., 2009). A special comment is earned to cartilage biopsy that is considered a critical raw material since it is the starting point for processing, or the cell source. Even if it is not the focus of this chapter, it is important to underline that

cartilage biopsy collection should be validated too in order to minimize microbial contamination, tissue amount and quality (to ensure a sufficient cell number) and possible impurities arising from fragments of the synovial membrane or bony tissue.

2.2 Chondrocyte isolation method

In the last years Researchers have developed several protocols with the aim to isolate chondrocytes from cartilage tissue. In general, they consisted of sequential enzymatic digestions that unbound cells from matrix entrapment. Although effective and repeatable, these methods revealed to be not always suitable for therapeutical applications. In particular, the use of animal origin enzymes and long lasting processing times revealed to be problematic. In fact, GMPs rules require both to avoid animal origin components that can be a source of zoonosis and to shorten product exposition times in order to minimize microbial contamination risks. The method traditionally used by our group for research comprises three sequential digestions including also animal origin reagents (Roseti et al., 2007). We translated this protocol into clinics using recombinant origin enzymes, reducing enzyme number and manipulation times.

2.2.1 Materials and methods

Healthy cartilage samples were harvested from the femoral condyle of three multi-organ donors (age: 23-71 years). To isolate chondrocytes three different methods were used.

A Method. Cartilage samples were minced with a scalpel and carefully washed with cell culture medium Dulbecco's Modified Eagle's Medium-GMP grade (DMEM) (Li StarFish, Carugate, Milano, Italia) supplemented with L-glutamine-GMP grade 4 mM (Li StarFish). The chondrocytes were then isolated by sequential enzymatic digestion: 30 minutes with 880 U/mL hyaluronidase (Sigma, St Louis, MO, USA) (10 mL/gr cartilage); 1h with 26.5 U/mL pronase (Sigma) (10 mL/gr cartilage) and 45 minutes with 740 U/mL collagenase II (Sigma) (20 mL/gr cartilage) at 37 °C, 5% CO_2 and humidified atmosphere. To block collagenase activity, DMEM supplemented with 10% Fetal Bovine Serum-Pharma grade (FBS) (Li StarFish) was added. The isolated chondrocytes were filtered by 100 and 70 µm sterile nylon mesh filters to remove cell raft and matrix debris. The filtrate was then centrifuged and the pellet washed twice. Viable staining assessed cell number and viability.

B Method. After mincing cartilage samples as described above (method A), digestion was performed only with 740 U/mL collagenase II (Sigma) (20 mL/gr cartilage) for 24 h at 37 °C, 5% CO_2 and humidified atmosphere. Isolation was then carried out as already described.

C Method. After mincing cartilage samples as described above (method A), digestion was performed only with 740 U/mL collagenase (Li StarFish) (20 mL/gr cartilage) for 24 h at 37 °C, 5% CO_2 and humidified atmosphere. Isolation was then carried out as already described.

2.2.2 Results

Comparison between the three methods, performed normalizing isolated cell number per cartilage gram, indicated C Method as the most efficient (Figure 1).

2.2.3 Discussion

C methods allowed to avoid animal origin enzymes, to reduce number of reagents (one enzyme instead of three) and manipulation times, giving at the same time the best yield results. Therefore, being the most GMP compliant, it was our choice as isolation method.

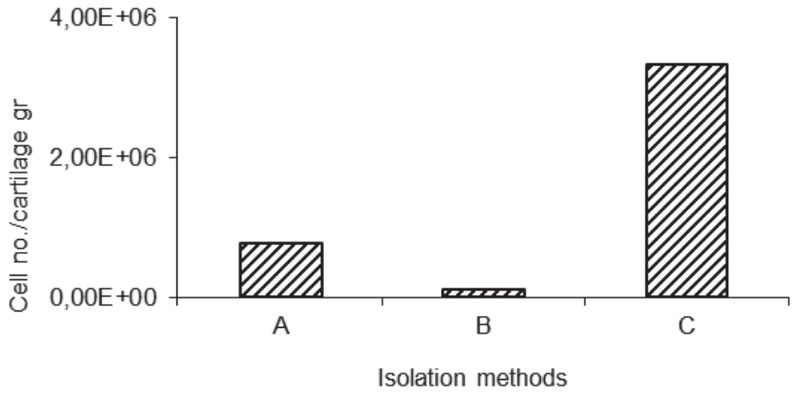


Fig. 1. Comparison of chondrocyte isolation methods.

A Method: sequential incubations with animal origin enzymes; B Method: animal origin collagenase II digestion; C Method: recombinant origin collagenase II digestion. Comparison was performed normalizing cell number per cartilage gram.

2.3 Chondrocyte monolayer expansion

Sera are mixtures of components essential for cell proliferation. They contain growth factors, hormones, molecules promoting cell adhesion and propagation, minerals trace and proteins like transferrin and albumin. FBS is traditionally and successfully used for cell cultures, including chondrocytes. The use of bovine-derived regents in clinical applications carries potential risks of contamination, especially Bovine Spongiform Encephalopathy (BSE) and immune responses. To develop our process we evaluated an alternative solution such as the use of human serum.

2.3.1 Materials and methods

C Method isolated chondrocytes (see section 2.2.1) were cultured in DMEM-GMP grade (Li StarFish) supplemented with L-glutamine-GMP grade 4 mM (Li StarFish) and with 10% FBS-Pharma grade (Li StarFish) or 10% human serum (HS) until passage two (three weeks). At 70% confluence cells were passaged with 1:250 Trypsin-EDTA-GMP grade (Li StarFish) and cell number and viability were assessed by viable staining. A high quality, FBS certified to be free of BSE and microbial, mycoplasma and viral contamination was utilized. Human serum was supplied in a sterile bag containing plasma drawn from donors. Under sterile conditions, plasma was added with calcium gluconate (0.3 ml/10 ml plasma) to induce coagulation process. After about two hours, the obtained serum was aspirated avoiding clots, transferred in a new bag and stored at -20°C.

2.3.2 Results

At passage 2, 10% FBS growth was three-times higher than 10% HS one (Figure 2).

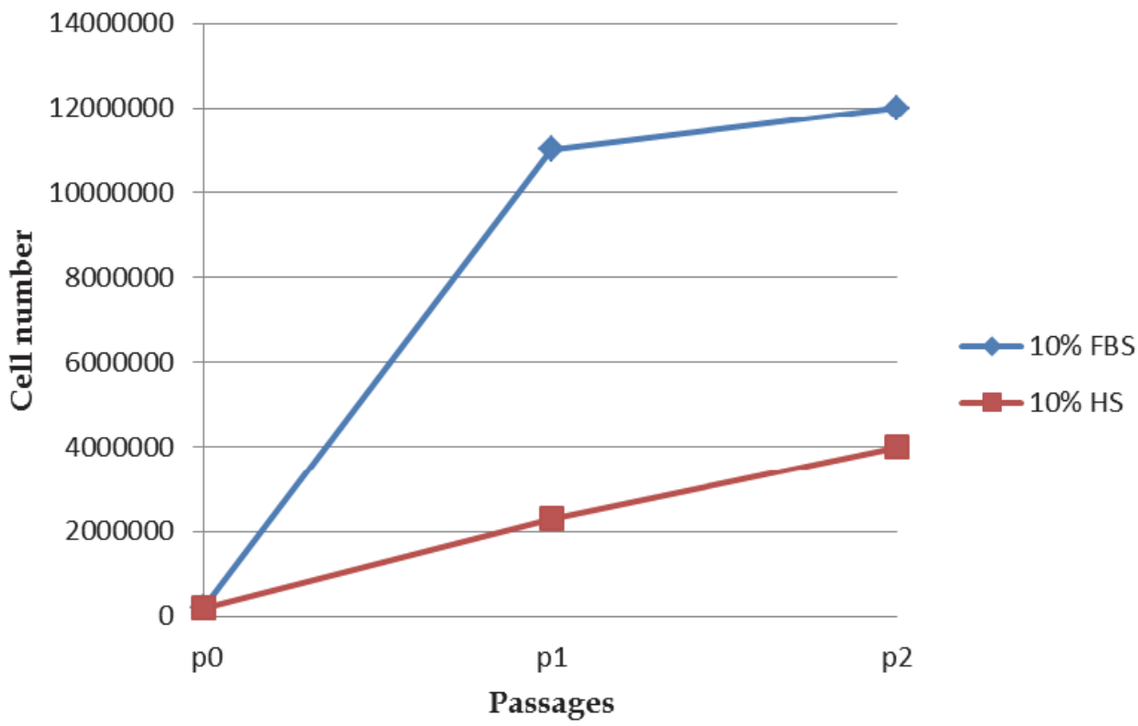


Fig. 2. Comparison of chondrocyte expansion methods. 10% FBS: expansion with 10%FBS; 10% HS: expansion with 10% human serum.

2.3.3 Discussion

The use of FBS for cell therapy is still controversial. Although bovine origin, FBS batches display less variability than the human ones and seem to better enhance cell growth. A reduction batch variability facilitate process standardization thus ensuring products consistency and robustness. Cell growth enhancement allows to shorten manipulation times and to reduce microbial contamination risks as well as progression to senescence. For these reasons our choice was towards FBS. The use of a high quality one (see section 2.1) appeared a good compromise between growth and safety requirements.

2.4 Cartilage engineered cultures

Many studies in the Literature have documented that monolayer expanded chondrocytes lose their phenotype becoming fibroblast-like cells. This de-differentiation process starts in the very first passages and progressively increases with time in culture. However, as attested by a number of study, such a situation can be reverted back when the cells are set in specifically defined conditions. It is as well documented that when de-differentiated

chondrocytes are seeded onto a biomaterial this specific configuration is able to allow the re-differentiation process to occur (Roseti et al., 2007). As already evidentiated, new ACI generations utilize different biomaterials as scaffolds onto which expanded chondrocytes can grow and re-acquire their original phenotype. The biomaterial used in our manipulation process is a matrix consisting of collagen without cross-linking or chemical additives (Chondro-Gide®, Geistlich Surgery, Germany). It is sterilized by gamma irradiation and provided in three different formats (2x3 cm^2, 3x4 cm^2 and 4x5 cm^2). The collagen is extracted from pig and purified to avoid immunological reaction risks. The membrane has a two-dimensional structure: a porous layer allowing cell seeding and culture and compact one acting as a barrier to prevent cell loss in the articular cavity. At the time of implantation the membrane is placed with the porous layer facing cartilage defect and the compact one facing the joint. Pre-clinical studies demonstrated its biocompatibility, low antigenicity, hydrophilicity (due to collagen fiber microstructure) and biodegradability. This membrane has already been used by other groups for ACI (Haddo et al., 2004). Based on their experience and on ours with other biomaterials we started to verify effectiveness of this biomaterial in allowing cell colonization. Due to problems related to FBS for clinical use, we cultured cartilage engineered constructs in medium without this reagent.

2.4.1 Materials and methods

Chondrocyte cultures were carried on in monolayer for three weeks, in the standard conditions described above (C Method for isolation; DMEM with FBS for expansion). After trypsinization pellets were re-suspended in DMEM without FBS. Chondrocytes were then seeded onto collagen membranes at concentration of $1x10^6$ cells/cm^2 surface. Cells were then let to adhere for 15 minutes and finally DMEM without FBS was added to cover the membranes. The loaded scaffolds were incubated at 37° C, 5% CO_2, and humidified atmosphere for 7 days. Cartilage engineered constructs were included in OCT and frozen at -80° C until analysis. Frozen blocks were cut in 25 mμ cryostat slices and slides were thawed and fixed in 4% paraformaldeid (PFA) for 30 minutes at room temperature. The samples were then rehydrated in H_2O for 10 min. Slides were incubated with hematoxylin for 1-2 minutes and, after 4 washes in distilled water, hematoxylin was activated under running water for 10 minutes. Slides were incubated with eosin for 5 minutes and washed four times in distilled water. After dehydration, slides were mounted with Entellan and stored at room temperature. Samples were analysed using a Zeiss Axioscope Microscope (Carl Zeiss, Oberkochen, Germany).

2.4.2 Results

Chondrocytes cultured on collagen-based membranes had a spherical appearance when observed by light microscopy (Figure 3) and were uniformly distributed among collagen fibers in the porous layer.

2.4.3 Discussion

Our data confirmed the ability of the collagen-based bilayer membranes that we intended to use in our process to allow cell colonization. The observed distribution pattern has been already evidentiated in other scaffolds displaying similar composition and structure (De Franceschi et al., 2005). Interestingly, FBS absence did not compromise viability and colonization ability.

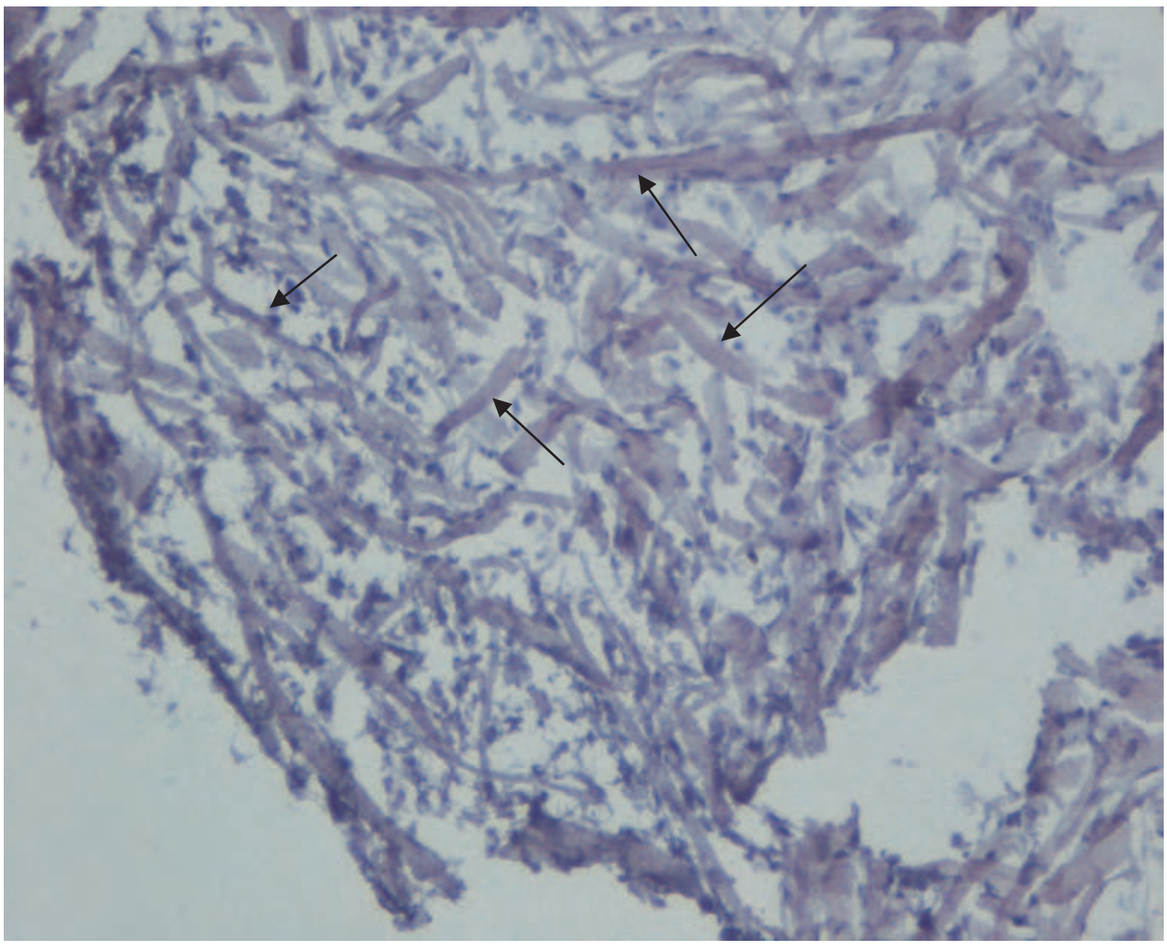


Fig. 3. Hematoxylin and eosin staining of porous layer in cartilage engineered tissues (40X). Spatial cell distribution is homogeneous among collagen fibers. The image is from one representative engineered tissue. Collagen fibers are indicated by black arrows.

3. Final products validation: identification of defined "specifications"

Final products of this GMPs manipulation model consist of engineered tissues derived from the combination of chondrocytes and bi-layer scaffold membranes. Validation is a GMPs requirement aimed at standardizing and thus defining final product peculiar features, technically named "product specifications". They allow the identification of a specific product obtained with a specific process and intended for a specific clinical use. Once defined, specifications cannot be changed. Products displaying even one different feature has to be considered "other" and cannot be released for the intended clinical use. To change product specifications a new re-validation process has to be performed. The first validation to achieve for a cell-based product is sterility. It is aimed at defining absence of aerobic and anaerobic bacteria, fungi, mycoplasma and endotoxins by means of specific analytical methods. It has to be underlined that it also mandatory that these analytical methods have been upstream validated in compliance with current European Pharmacopoeia and GMPs. Besides sterility, that is not the focus of the current chapter, the other specifications needed for cell-based products are: viability, potency, purity, yield and stability. To perform final

product validation we utilized the same cells and protocols as for process validation and analyses were performed onto the obtained cartilage engineered tissues.

3.1 Viability

In the Literature there are many methods for viability evaluation. Difficulties for analysis arise when cells are entrapped into a matrix or a scaffold, like in our process. In this case there is a real risk to underestimate the results. The Authors had already faced with this problem when managing other types of chondrocyte-scaffold constructs (Roseti el al., 2007). In that occasions they were able to standardize a feasible method also applied in this model.

3.1.1 Materials and methods

Three engineered tissues -i.e. chondrocytes and collagen-based membranes (see section 2.4.1)- and named Case 1, Case 2 and Case 3 were analysed. Cell viability was determined at the seeding onto the biomaterial (Day 0) and after 7 and 14 days in culture by 3-(4,5-dimetiltiazolo-methyl)-2.5-difeniltetrazolio bromide (MTT) (Sigma)-mitochondrial reduction method, based on Mosmann original protocol (Denizot & Lang, 1986). Briefly, engineered constructs were transferred to 35x10 mm Petri dishes, added with 1 ml of a solution of 1 mg/ml MTT in PBS 1X and incubated for 3 hours. The membranes were then transferred in Eppendorf tubes and added with 1 ml of extraction solution consisting of 0.01N HCl in isopropanol. The membranes, still contained in the Eppendorf tubes were then shaken and centrifuged at 14,000 rpm for 5 min to allow complete solubilisation of formazan. Finally, supernatant absorbance was read at 570 nm using a Beckman spectrophotometer.

3.1.2 Results

MTT testing, which is directly related to chondrocyte activity, showed slight increased values until day 7 for Case 1 and 2 and then a plateau maintained until day 14. Case 3 chondrocytes did not show increased values, but immediately reached a plateau (Figure 4).

3.1.3 Discussion

The engineered constructs showed slight or no chondrocyte growth. These results are in contrast with the ones obtained with other biomaterials (Roseti et al., 2007) that highlighted a cell proliferation increase by time. We believe that this is to be due to FBS deprivation that, however, allowed viability maintenance until day 14. The choice to use FBS only for the monolayer expansion phase where we demonstrated its efficacy in favouring cell growth (see section 2.3) and not for the engineered tissues represents for the Authors a good compromise to guarantee a safer therapy.

3.2 Potency

For chondrocyte-based products potency can be defined as cartilage forming capacity (Reflection paper on *in-vitro* cultured chondrocyte containing products for cartilage repair of the knee. Final. London, 08 April 2010 EMA/CAT/CPWP/568181/2009 Committee for Advanced Therapies [CAT], 2010). Therefore, to investigate if the final products displayed cartilage features we evaluated the presence of collagen type II and aggrecan, that are main recognized markers of this tissue (Becerra et al., 2010).

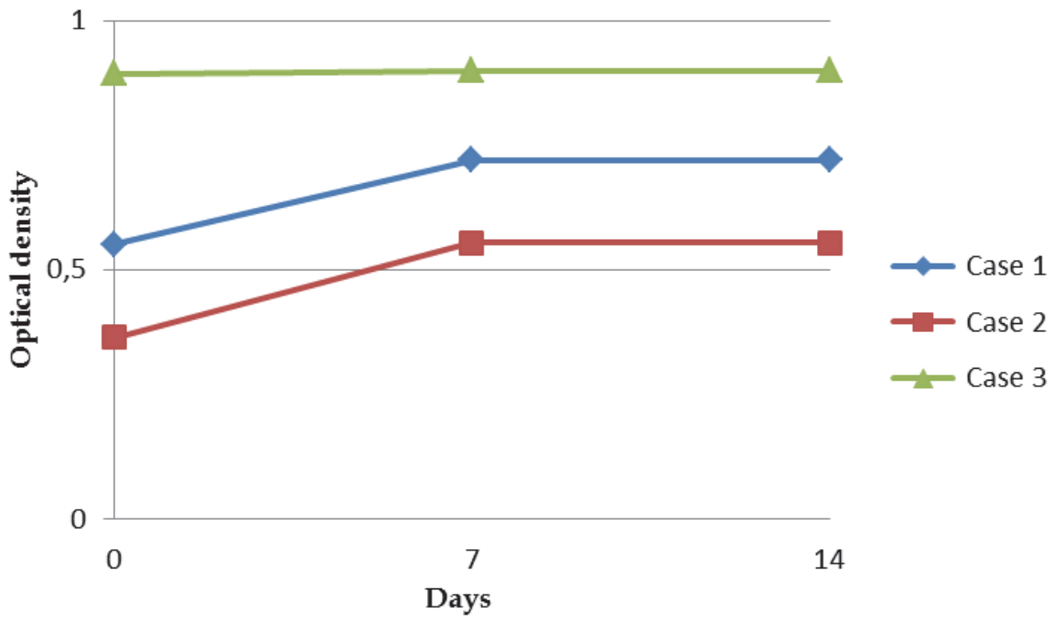


Fig. 4. MTT testing on engineered tissues (chondrocytes and collagen-based membranes) indicated as Case 1, 2 and 3. Samples were analysed after 0, 7 and 14 days in culture. Data are expressed as optical density at 570 nm.

3.2.1 Materials and methods

Tree engineered tissues -i.e. chondrocytes and collagen-based membranes (see section 2.4.1)- were embedded in OCT, snap-frozen in liquid nitrogen, cut into 25 μm sections, air-dried and stored at -20 °C until use. These slides were transferred at room temperature, air-dried for 20 minutes and fixed in 4% PFA at room temperature for 20 minutes. The following primary antibodies were used: mouse anti-human collagen type II monoclonal antibody and mouse anti-human proteoglycans (Chemicon International Temecula, CA, USA). Air-dried fixed samples were rehydrated. Slides for collagen type II determinations were treated with 0.1 % hyaluronidase (Sigma) in Phosphate Buffered Saline (PBS) at 37°C for 5 minutes for epitope unmasking; those for proteoglycans with chondroitinase ABC (Sigma) in Tris-HCl pH=8 for 30 minutes at room temperature. The slides were then incubated with the primary antibodies diluted 1:40 (collagen type II) or 1:50 (proteoglycans) in 0.04M Trizma Base Saline (TBS) pH 7.6 containing 1% BSA and 0.1 % Triton X-100 for 1 hour at room temperature. After washes in PBS with the addition of 1% BSA, the slides were incubated with biotinylated immunoglobulins specific for various animal species (BioGenex, San Ramon, CA, USA) for 20 minutes at room temperature. Then samples were incubated with a phosphatase-labeled streptavidin (BioGenex) for 20 minutes at room temperature, and after washes the reactions were developed using fast red substrate (BioGenex). Negative controls were performed by omitting the primary antibody. Slides were counterstained with hematoxylin and mounted in glycerol gel. All the samples were analysed using a Zeiss Axioscope Microscope (Carl Zeiss).

3.2.2 Results

The engineered tissues revealed to be able to re-express specific cartilage markers. In particular, collagen type II immuno-staining revealed the presence of homogeneously diffused positive cell clusters (Figure 5) while proteoglycans appeared to be distributed in the extra-cellular matrix (Figure 6).

3.2.3 Discussion

This potency validation allowed to define an identity for our products, as specifically required by chondrocyte-based medicinal products guidance. It has to be mentioned that, as evidentiated in the Literature (Saris et al., 2008), one limitation of this system could be the inability to quantify results thus avoiding product good quality ranking. The Authors, on the other hand, suggest that such a quantification could not be really indicative of product quality or positive outcome in patients since there is still not complete evidence of a direct correlation between these features and because of documented patients variability in terms of basal values of cell number and cartilage markers expression.

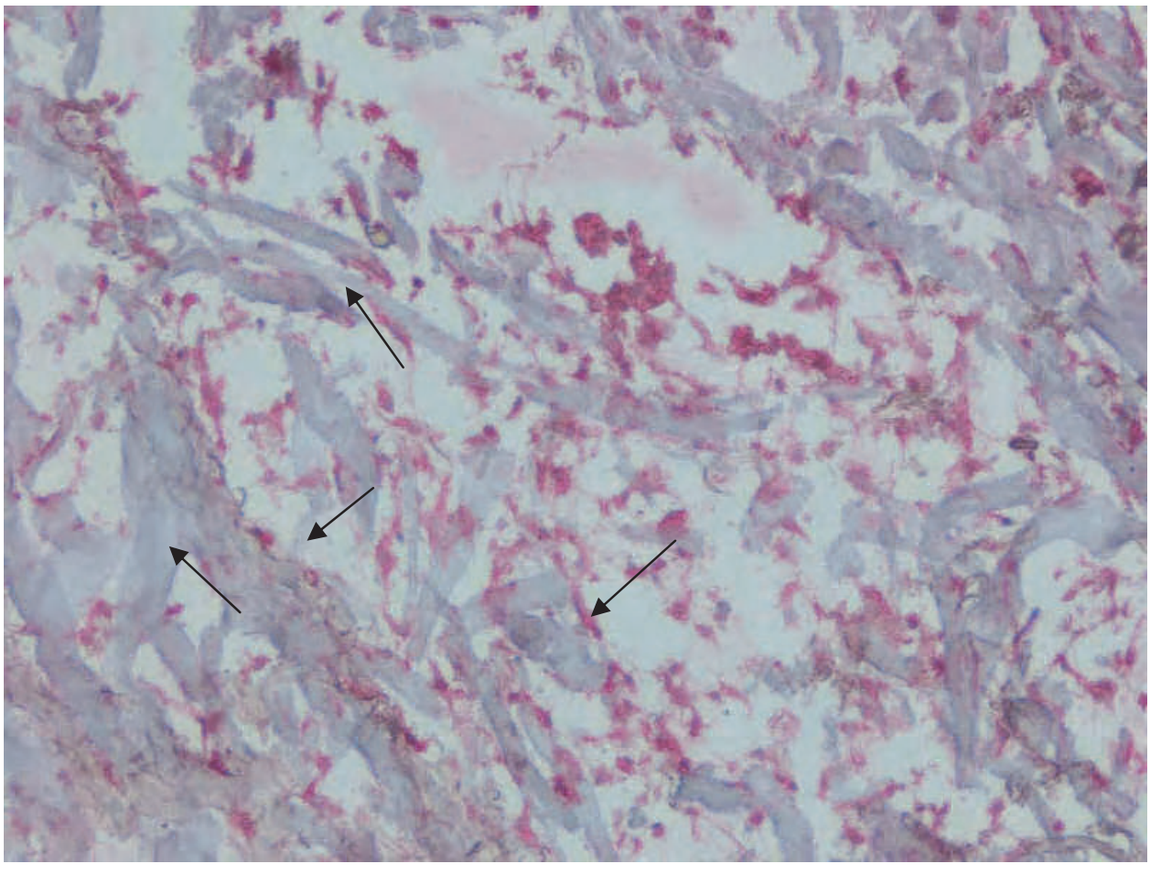


Fig. 5. Collagen type II immuno-staining on engineered constructs (80X).The image from one representative sample shows that the protein is uniformly distributed and mainly located inside the cells or in the peri-cellular matrix. Staining was developed using fast red substrate (red is positive stain). Biomaterial fibers are indicated by arrows.

3.3 Purity

Purity is typically required for drugs. For advanced cell therapy medicinal products it means elimination or decrease of undesired cells. The unique type of cells in cartilage is chondrocyte. However cartilage biopsy should carry possible contaminants arising from fragments of the synovial membrane or from bone. These contaminants could be maintained during culture and thus become a part of the final products. To minimize these risks we standardized biopsy collection (data not shown) and developed a chondrocyte-specific culture method. Nevertheless, to be fully compliant with GMPs, we had to give evidence that our final products were free of cell contaminants. Therefore we analyzed the engineered tissues for the presence of two markers, one typical of fibroblasts (collagen type I) and one of bone phenotype (osteocalcin).

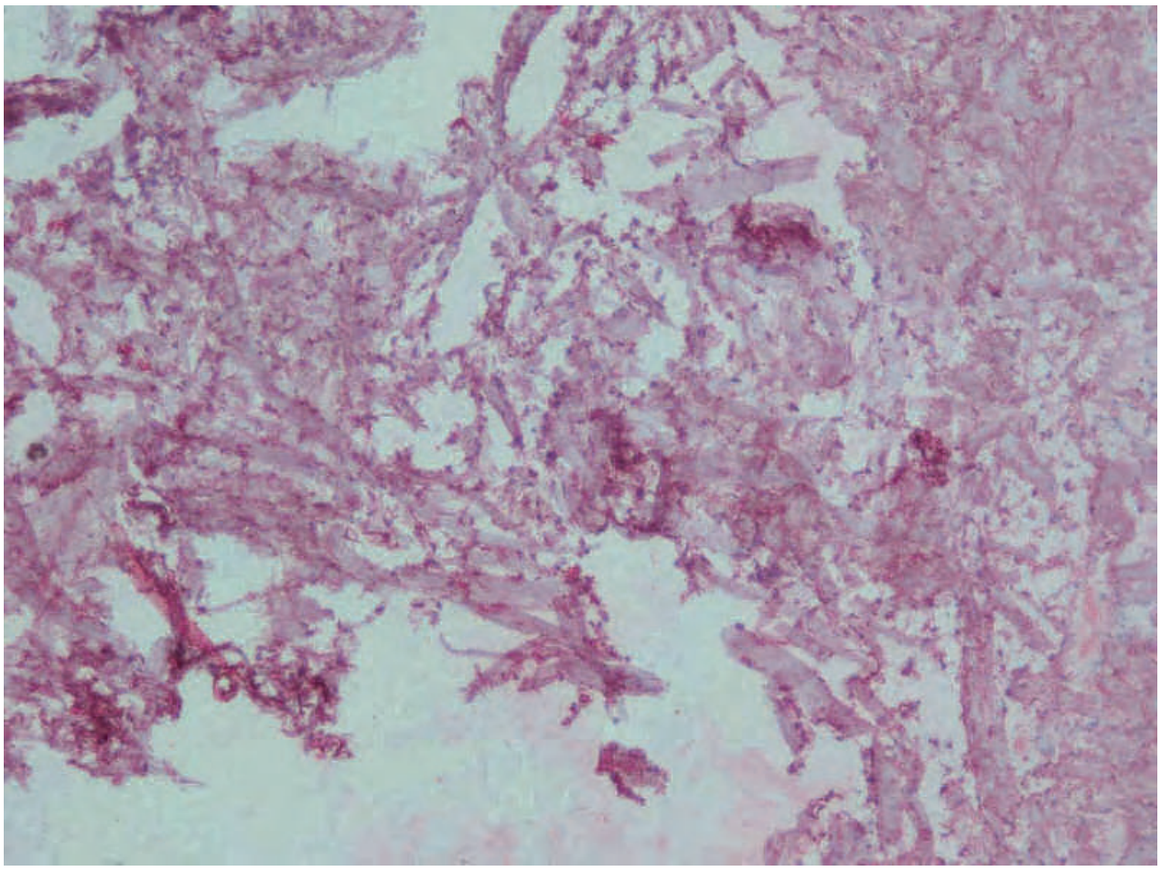


Fig. 6. Proteoglycans immuno-staining on engineered constructs (40X). The image from one representative sample shows that proteoglycans were homogenously distributed throughout the whole extra-cellular matrix. Staining was developed using fast red substrate (red is positive stain).

3.3.1 Materials and methods

The same procedure described for potency validation was utilized (see Section 3.2.1). The following primary antibodies were used: mouse monoclonal anti-human collagen type I

(Chemicon International) diluted 1: 20 and Mouse anti-human osteocalcin (R&D Systems, Inc., Minneapolis, MN, U.S.A.) diluted 1:40. Slides for collagen type I determinations were treated with 0.1 % hyaluronidase (Sigma) in PBS at 37°C for 5 minutes for epitope unmasking.

3.3.2 Results

Engineered constructs were negative for collagen type I (Figure 7,) and osteocalcin (Figure 8) immuno-staining both in the cells and in the newly synthesized matrix.

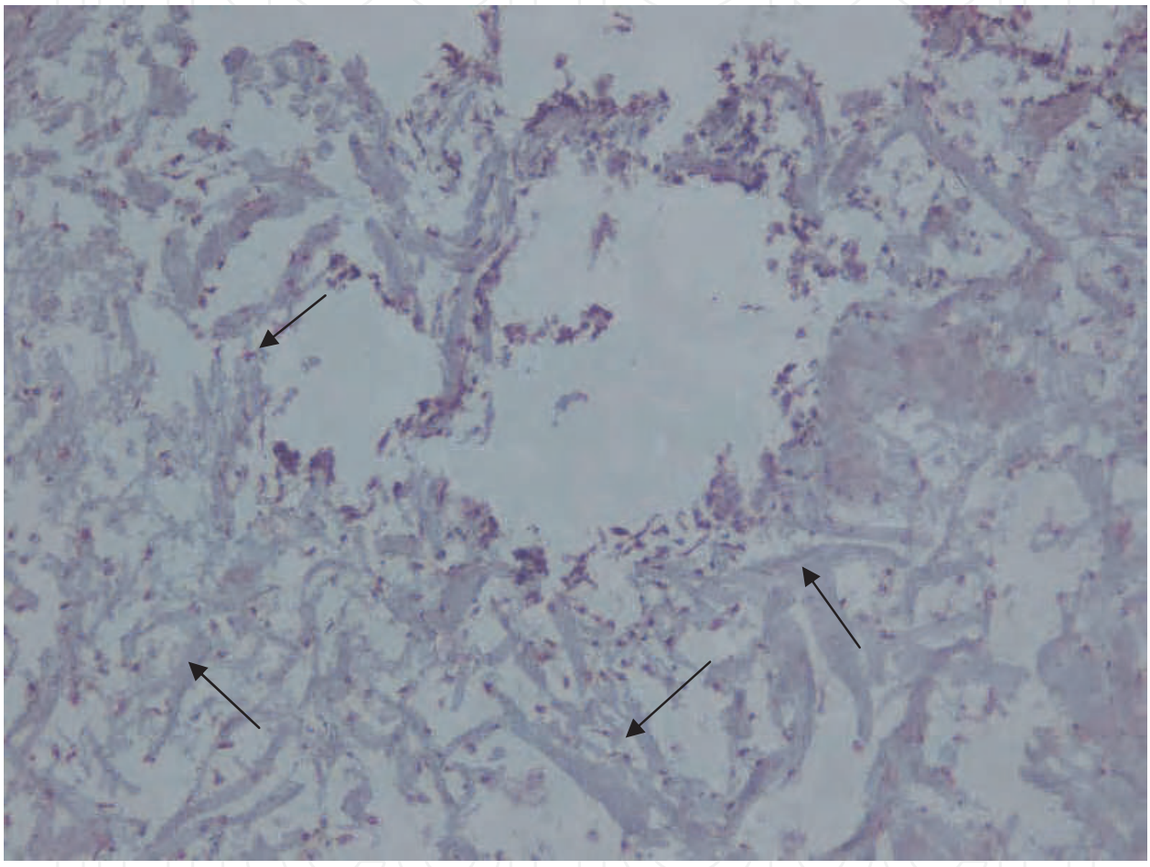


Fig. 7. Collagen type I immuno-staining on engineered constructs (40X). The image from one representative sample shows that the protein is not present (no red areas were observed using fast red substrate). Biomaterial fibers are indicated by arrows.

3.3.3 Discussion

This validation is a further evidence of a defined identity of our products. Collagen type I absence is particularly meaningful: on one hand it indicates that synovial cells or tissues were not present at biopsy harvest and/or were not carried on with cultures; on the other hand it highlightes that chondrocytes within this biomaterial had reverted back to their original phenotype that was lost in monolayer culture.

3.4 Yield

Yield can be defined as the number of cells obtained for each medicinal product. GMPs guidance requires yield validation, but in our experience this can be difficult to perform. In fact, yield is, more than the other specifications, subjected to variables that make quite problematic the required standardization. Intrinsic variability due to patient, cartilage quality or cell growth capacity do not completely depend from operators or process conditions and cannot be totally controlled. When we started our process we had yield validation data allowing a cell seeding onto the biomaterial of $1x10^6$ cells per cm^2. Lately, after more than 90 cultures, this initial specification has been replaced by a range of values: $0.1 \div 1.1x10^6$ cells per cm^2. To accept and apply this new yield scale we had to re-validate the process and the products verifying that each value of the range was able to guarantee the development of viable and cartilage-like engineered tissues.

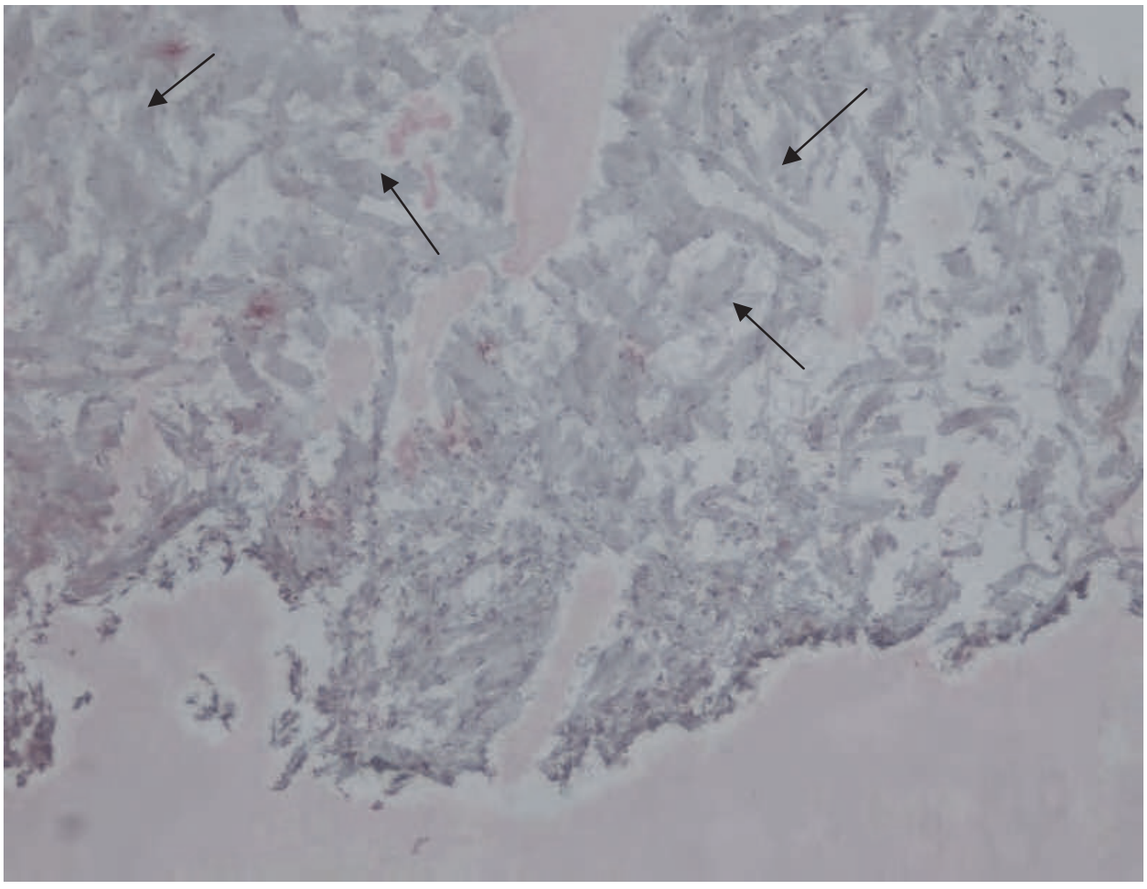


Fig. 8. Osteocalcin immuno-staining on engineered constructs (20X). The image from one representative sample shows that the protein is not present (no red areas were observed using fast red substrate). Biomaterial fibers are indicated by arrows. Biomaterial fibers are indicated by arrows.

3.5 Stability

Process validation ensures the manufacturing of standardized products displaying defined characteristics. But what happens after product release? A critical point is product stability over time: cell manufacturers should find the best storage conditions and times ensuring maintenance of products specifications. To define a product "shelf life" we performed a research in the literature and in the Companies/Institutions already producing this type of cells. Then we evaluated our products for each specification (see section 3) at release and at different times after. As expected, quality products started to decrease from the first day, but we found that after 4 days each specifications was preserved for at least 70% of the initial value. Therefore we chose that period of time as the shelf life of our products.

4. Cryopreservation phase

Chondrocyte manufacturing process may require a cryopreservation step in liquid nitrogen if, for any reason (technical or clinical) the ACI intervention is delayed or if a reservoir of cells is requested by the surgeon for other future treatments in the same patient. In both cases it is required that aliquots of cells are stored as "intermediate products" in proper and defined conditions. A validation step should be performed in order to demonstrate that the freezing/cryopreserving/thawing process does not alter all the cell properties needed for implantation. Considering our experience and literature data we decided to use dimethyl sulfoxide (DMSO) as cryo-protector agent.

4.1 Materials and methods

Three chondrocytes cultures (C Method isolation) at passage 1 were frozen and stored in liquid nitrogen gases. The freezing solution utilized was: DMEM-GMP grade (Li StarFish) supplemented with FBS-Pharma grade 40% and CryoSure-DMSO (Li StarFish) 10%. Samples were then thawed and cells seeded in medium supplemented with 20% FBS. After seven days, cultures returned to usual standard medium conditions (10% FBS). Chondrocytes were then seeded and cultured onto collagen biomaterial as described above. The final products were the checked for viability, yield, potency, purity and stability.

4.2 Results

In the three cultures analyzed the thawing process allowed a survival of 70, 92 and 70% of the cells, respectively. Cells were able to proliferate in monolayer and to reach a number sufficient for the seeding onto the collagen-based biomaterial. The final products revealed to be similar to the ones obtained without the cryopreservation step (data not shown). In particular, cells were viable, and expressing only typical cartilage markers.

4.3 Discussion

We developed a cryopreservation phase that can be included in ACI procedures without altering characteristics of final cartilage constructs.

5. Conclusion

The transfer technology described in this chapter allowed the development and validation of a safe, effective and robust GMPs compliant process articulated in different steps and

including a potential stand-by phase in liquid nitrogen. This process results in chondrocyte-based medicinal products (a combination of cells and collagen-based membranes) with defined identity and stability that make them suitable for ACI therapeutical option in patients with articular cartilage damages. The Authors haver three main comments to disclose, based on this experience. The first is about the use of FBS in culture which, besides animal origin related problems, may imply immune responses in patients. We justify our choice to use FBS in monolayer conditions because it allowed a better cell growth standardization. However, since its potentially dangerous action could not be ignored, we decided to avoid FBS presence in the final products. This resolution was supported by validation data that were showing that the engineered tissues were cartilage-like also without this supplement. These results revealed to be in line with the literature and with data obtained by the Authors themselves with other chondrocyte constructs. Only the ability to grow inside the collagen-based biomaterial was importantly reduced, even if viability was consistently maintained. Therefore, considering implications due to FBS presence, our choice could be a good compromise for patient's safety. The second comment is about the validation procedure itself. GMPs Guidance gives strict indication on how to carry on process and product validation thus minimizing contamination risks and variable elements. However such a standardization could be difficult to apply for cells. It is known that cell characteristics in culture are labile and subjected to modulation due non only to culture conditions (times, culture media, supplements an scaffolds) but also to patients (age, gender and pathology). In particular, quantification of some cell properties can become really hard to perform, thus hampering standardization. Therefore validation of a cell-based medicinal product, should mediate between the required "drug rules" and the intrinsic well known cell biological variability that is impossible to eliminate. The last comment is a consideration that cell therapy for cartilage regeneration is under vast exploration and there are now emerging other possibilities as well as improvement in this application. Allogeneic implantation, unexpanded chondrocytes, cell combination with new scaffolds and the use of pre-committed or undifferentiated precursors or mesenchymal stem cells from different sources are some examples of recent advancement in this field. In any case, Country legislation must be applied and our system can be also assumed as a valid model for the compliant GMPs development of other new products suitable for clinical purposes.

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentials of different synthetic and engineered biomaterials. Contributions were not selected based on a direct market or clinical interest, than on results coming from very fundamental studies which have been mainly gathered for this book. This fact will also allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The book collects 22 chapters related to recent researches on new materials, particularly dealing with their potential and different applications in biomedicine and clinics: from tissue engineering to polymeric scaffolds, from bone mimetic products to prostheses, up to strategies to manage their interaction with living cells.

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