Cartilaginous tissue has limited capacity for regeneration of lesions associated with it, since the natural repair process leads to the formation of fibro-cartilaginous tissue which does not have the resistance and capability of deformation under load, typical of hyaline cartilage which covers the articular surfaces. The possibility of transplanting human chondrocytes for cartilage reconstruction has been demonstrated in orthopaedics.

The scope of our study is to evaluate the possibility of cultivating and expanding human chondrocytes and seeding them on pure equine type I collagen support.

Our results show that human articular cartilaginous cells can multiply and grow on type I collagen substrate with production of extracellular matrix. This type of chondrocyte culture on a support can be used for repairing cartilaginous lesions since they show a correct morphology (evaluated by cytological and histological methods) and a suitable differentiation and phenotype as shown by Alcian PAS staining to indicate the
presence of mucopolysaccharides, and immunohistochemical methods to identify collagen II. The use of scaffolds ought to lead to improvement of the surgical technique, making it possible to hold the cells “physically” in the area to be repaired and malleable use to allow optimum spatial adaptation inside injuries of all shapes.

INTRODUCTION

Hyaline cartilage is a highly specialized tissue derived from the mesenchyma during embryonic development\[14\]. Like all connective tissues, it is composed of cells (chondrocytes) and the intercellular substance (formed by the chondrocytes) which is in the form of a gel and consists of two components: collagen fibres and a fundamental or amorphous substance rich in protein–mucopolysaccharides complexes (proteoglycans). These compounds are large polymers made up of about 95% polysaccharides and about 5% proteins\[13–36\], in other words, a protein core to which a number of disaccharide units called glycosaminoglycans (sulphated acid mucopolysaccharides) are attached laterally by means of covalent bonds; the main glycosaminoglycans in cartilage are chondroitin sulphate and keratan sulphate\[36\].

In the extracellular matrix, most of the proteoglycan molecules are linked to a long hyaluronic acid molecule by means of a protein bond, thus forming proteoglycan aggregates (or aggrecans); a single hyaluronic acid molecule can have up to
100 proteoglycan molecules; these long compounds interact
with the collagen fibrils by means of electrostatic bonds\textsuperscript{[36]}.

Type II collagen forms about 90 – 95 % of the total collagen
content of hyaline cartilage, and this is why it is usually used as
the marker for this type of cartilage\textsuperscript{[13–19–27–31]}. The other types of
cartilage are V, VI, IX, X, XI\textsuperscript{[13–36]}.

The main function of hyaline cartilage is to facilitate sliding of
articular surfaces covering and transferring the weight to the
underlying bone\textsuperscript{[36-39]}. When damaged, cartilage has poor
capacity for regeneration\textsuperscript{[6–31]} because of the absence of blood
vessels and innervation\textsuperscript{[41]}; other factors that explain the poor
capacity for self-repair of cartilage are:

- poor metabolic activity;
- inability of resident chondrocytes to migrate to the defect
  because of the presence of extracellular matrix;
- poor cellularity\textsuperscript{[8-31-36]}.

The natural repair process leads to the formation of fibro-
cartilaginous tissue [1] which does not have the resistance and
capability of deformation under load typical of hyaline cartilage,
which are due to its biochemical composition\textsuperscript{[13-31]}.

The need to find strategies for repairing articular cartilage is an
important achievement, if we consider the frequency of traumas
and injuries this tissue is subjected to, following road accidents
or sports accidents as well as the high percentage of
inflammatory, rheumatic and degenerative diseases the
cartilage has to face with ageing\textsuperscript{[1–13-36]}.

The estimated incidence of osteoarthritis of the knee in the
world is about 30 million cases and about 1.2 million focal
defects\textsuperscript{[25-31]}. 
Numerous treatment protocols were proposed for trying to repair cartilaginous tissue, but the results, especially long-term, were unsatisfactory\cite{27-39}.

Tissue engineering is a multi-disciplinary area of research which has the aim of regeneration of damaged tissues and organs in human body, starting from the assumption that almost all animal tissues can be cultivated in the laboratory. The general principle is to take stem cells from the patient who requires the transplant, allow them to grow and differentiate on a suitable support to produce the tissue that needs replacement\cite{13–30}.

The cartilage formation process by means of tissue engineering has three objectives\cite{25–27–37-41}.

The first is to increase the number of cells to reach the quantity necessary to repair the cartilaginous defect\cite{4-40}. Chondrocytes can be obtained from small amounts of cartilage removed arthroscopically from areas of the knee not subjected to load, or from damaged articular cartilage removed during routine arthroscopic surgery, from the base and sides of the lesion\cite{5-6}. It is important to bear in mind that 1 mg of cartilage will give approx. 2,500 cells\cite{4-5-6}, therefore 300 to 500 mg are necessary.

Once freed from their matrix by enzymatic digestion and cultivated in monolayer at low density (10,000 – 30,000 /cm\(^2\)), chondrocytes rapidly lose their rounded morphological appearance and the biochemical features, and take on a fibroblastic phenotype and appearance and divide actively\cite{2-4}.

In place of collagen II, chondrocytes synthesize collagen I \cite{6-19-}. ...
cartilage oligomeric matrix protein)\textsuperscript{[7]} with reduction of aggrecans\textsuperscript{[14]}. Cartilaginous cells can also be obtained using pluripotent cells, especially mesenchymal stem cells which are capable of differentiating into bone, cartilage, tendons and muscles\textsuperscript{[25]}; other cells with chondrogenic and osteogenic potential have been isolated from various tissues such as the periostium, bone marrow, spleen, skeletal muscle, adipose tissue, skin and the retina\textsuperscript{[10-25-27-31]}. A new population of chondro-progenitor cells was recently isolated from the surface area of articular cartilage\textsuperscript{[25]}.  

The second objective is to obtain the correct phenotype\textsuperscript{[39]}. The mature chondrocyte is morphologically characterized by its rounded or oval shape with little eosinophilic cytoplasm and the nucleolus not very evident in the nucleus. The chondrocytes are contained in optically empty spaces called lacunae which condense at the periphery to form the capsule; this is surrounded by plenty of extracellular matrix, characterized by the presence of type II collagen fibres\textsuperscript{[9]} immersed in a fundamental or amorphous substance containing proteoglycans\textsuperscript{[1]}.  

The third objective is the three-dimensionality of the product\textsuperscript{[33]}, bearing in mind that the average thickness of articular cartilage is approximately 1.5 mm\textsuperscript{[31]} and the size must suit the type of articular defect. Transplanting chondrocytes or undifferentiated cells to restore missing tissue can be done by means of cells in suspension or seeded on support.
As regards the first method, a Swedish study published in October 1994 in the New England Journal of Medicine showed that it is possible to regenerate hyaline cartilage by autologous chondrocyte transplantation\cite{3}. The technique consists in growing the patient’s cells in the laboratory and reapplying it in suspension inside the defect, using a periosteal flap sealed airtight to hold it in place to prevent dispersal in the synovial capsule. The cells adhere to the underlying bone and gradually regenerate a new cartilaginous tissue which, over time, assumes features similar to the original cartilage\cite{1-4}.

After the initial encouraging results, this procedure showed defects including the need for the patient to undergo complex surgery: it is, in fact, an invasive method; a study conducted by Breinan\cite{4} showed that at 12 – 18 months following surgery there were no differences in the results between untreated control cases, cases treated only with the use of the periosteum, and cases treated with autologous chondrocytes and periosteum; other studies\cite{6} also showed (in 4 patients out of 10 with biopsies carried out during arthroscopy at 18 – 24 months after surgery) the presence of fibrocartilage or absence of healing.

The use of cellular components supported on suitable scaffolds may give positive results in repairing articular tissue, allowing improvement of the surgical technique. The use of scaffolds, in fact, makes it possible to improve cell implantation, reproduction and differentiation and “physically” hold the cells in the area to be repaired\cite{13}.

The biomaterials must have the following features\cite{23-25}:
• porosity: to allow cell growth and allow metabolic exchange,
• tolerability: must be immunologically inert (biocompatible),
• temporary scaffold: after integration, the biomaterial must be replaced by the original tissue (biodegradable),
• informative content: must communicate and exchange signals with the host cells\textsuperscript{[17]}.
These supports must also allow cell multiplication and matrix production while at the same time ensuring nutrition. Not less important in the case of chondrocyte cells transplantation, these scaffolds must allow re-expression of the original phenotype which is lost during expansion of the monolayer culture\textsuperscript{[36]}.
The most important factors for obtaining a correct phenotype of hyaline cartilage are high density seeding, culture time and O\textsubscript{2} tension\textsuperscript{[6–16]}.
Numerous materials were used, such as scaffolds for the growth and differentiation of chondrocytes\textsuperscript{[26-29–36]} especially collagen\textsuperscript{[8–9–12–19–20–24–34]}, agarose\textsuperscript{[5]}, hyaluronic acid\textsuperscript{[4–13–23]}, fibrin glue\textsuperscript{[15–18–32–35]}, alginate\textsuperscript{[6]}, PGA\textsuperscript{[12–33]}, PHBV\textsuperscript{[20]}, PEG\textsuperscript{[21]}.
A great deal of attention is paid to collagen; studies have shown that when used as support, the collagen allows the chondrocytes to develop and differentiate in a suitable manner\textsuperscript{[4-5-40]}.

The aim of our study is to isolate human chondrocytes from hyaline cartilage, allow their multiplication in a monolayer and differentiation on a pure collagen type I support (ANTEMA\textsuperscript{©} SOFT, Opocrin S.p.A.), checking the exact morphology and phenotype by means of cytohistological and immunohistochemical methods.
MATERIALS AND METHODS

Removal of Cartilage was removed from the femoral condyle of a 63-year old male patient who underwent surgery for arthroprosthesis of the hip total knee replacement.

Laboratory procedure

Chondrocytes are isolated from the cartilage after it is finely chopped and washed with Dulbecco’s modified Eagle’s medium (DMEM) without FCS; it is then digested sequentially with hyaluronidase (Sigma, 0.1% at 37 °C for 30 minutes); Pronase (Sigma, 0.5% at 37 °C for 1h) and Collagenase (Sigma, 0.2% at 37 °C in a thermostated bath for 45 minutes in agitation). The cells thus isolated are re-suspended in the complete medium containing DMEM, antibiotic, L-glutamine and 10% of bovine fetal serum; seeded in 75 cm² cell culture flasks and maintained at 37 °C in a humidified environment with 5% CO₂.
The culture medium is changed every 3 days. When the cells are at semiconfluence the flask is incubated with 0.05% trypsin and 0.02% EDTA for 8 minutes; the cellular suspension obtained is centrifuged at 1800 rpm for 7 minutes and the pellet obtained is re-suspended in the medium. The viability and number of cells is evaluated by staining with Trypan Blue and haemocytometer.
The cells can be directly used for seeding on scaffold or preserved by freezing first at −80° and then in liquid nitrogen. The chondrocytes are seeded on a 0.15 cm Type I, commercial collagen sponge (ANTEMA® SOFT, Opocrin S.p.A, Modena, Italy).
ANTEMA® SOFT, a lyophilized 1.5 mm thickness sponge, is made of pure type I collagen, extracted from equine tendons and purified according to a manufacturing method which removes immunogenic structures (telopeptides) from the protein.

The scaffold is first washed with saline solution and then with PBS to obtain pH 7. The chondrocytes are then seeded at a density of $1 \times 10^6$/cm$^2$ in 100 µl of medium, for a 3 cm$^2$ membrane and a maximum of 3 ml for a 100 cm$^2$ membrane. About 3 hours after seeding, the membrane is completely immersed by adding enough medium. The culture medium is changed every 3 days.
HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

Every three days, a fragment of the support with chondrocytes, approx. 1 cm in size, is taken in sterile conditions and immersed in fixative liquid (10% formalin) for a minimum of two hours. The sample is then subjected to dehydration, clarification and inclusion. Dehydration is done by passing the sample through a series of alcohol concentrations progressively increasing from 70% to 100%, for times varying from 40 minutes to one hour. The next phase is clarification in xylene. The pieces are then embedded in paraffin allowed to solidify in special moulds.

3-micron microtome sections of the sample are taken which are then spread out in a thermostated water bath (37°C) and then collected on histology slides. The slides are dried, the paraffin is removed first by treatment with xylene and then with a decreasing series of alcohol concentrations (100%, 95%, 50%). Sections are then re-hydrated by passing through water.

A section is stained with Haematoxylin-Eosin (H.E.). This is a routine staining procedure based on the staining affinity of the cellular structures for various stains: aqueous solutions of Haematoxylin (basic) stain the nuclei (containing nucleic acids) purplish-blue and alcoholic Eosin solutions (acid) stains the cytoplasm (rich in basic proteins) red-orange. The section is immersed in haematoxylin for 16 minutes, washed in tap water, immersed in 95% alcohol, stained with alcoholic eosin, dehydrated in alcohol, clarified in xylene, and mounted with balsam.

To highlight the production of extracellular matrix, one of our sections is stained with Alcian-PAS. After staining in Alcian Blue
pH 2.5 for thirty minutes, the section is washed in tap water, then treated with 0.5% Periodic Acid for 10 minutes, washed with tap water for five minutes; then treated with Schiff’s Reagent for fifteen minutes; washed in tap water; counter-stained with Carazzi haematoxylin for three minutes; washed in water, clarified and then the slide is mounted with balsam. The Alcian Blue stains the acid mucosubstances blue-turquoise; the PAS stains the neutral mucosubstances and carbohydrates magenta red and the nuclei blue.

Immunohistochemical staining procedures allow the visualization of antigen by sequencing an antibody specific to the antigen (primary antibody), a secondary antibody, which binds the primary antibody, and chromogenic substrate. The application of the three reagents alternates with washing phases. The enzymatic activation of the chromogenic substrate produces a reaction visible on the antigenic site. The results are read using an optical microscope.

The following antibodies have been used in our study: alfa-actin (Cell Marque, clone 1A4), CAM 5.2 (Becton Dickinson, 1:2), CD 57 (Cell Marque, clone NK-1), CD99 (Cell Marque, 1:80), collagen IV (Cell Marque, clone CIV 22), Keratin AE1 (Ventana Medical System), Keratin AE3 (Cell Marque, clone 34 beta E12), NSE (Cell Marque, clone E27), S100 (Ventana Medical System), vimentin (Ventana Medical System, clone V9).

In particular the production of Collagen II was detected by means of immunohistochemical studies, using the avidin-biotin system.

The technique consists of the following phases:
1) inhibition of endogenous peroxidase and incubation for 4 minutes in a chamber heated to 37°C;
2) antigen unmasking with an enzymatic solution (protease 1);
3) application of anti-collagen II monoclonal primary antibody (clone 6B3; NeoMarkers) diluted 1:80 with diluent; incubation for 32 minutes;
4) application of polyclonal secondary antibody conjugated with biotin; incubation for 8 minutes;
5) application of avidin-peroxidase compound; incubation for 8 minutes;
6) application of chromogenic-substrate (hydrogen peroxide - diaminobenzidine); incubation for 8-10 minutes;
7) washing with water;
8) counter-staining with haematoxylin for 4 minutes.

The slide is then washed, dehydrated, clarified, mounted.
RESULTS

The extraction of chondrocytes obtained in our experiment from cartilage fragments made it possible to obtain 2 million cells, following seeding in an F 75 flask. 

48 hours after seeding, the cells observed under the phase contrast microscope appear branched with cytoplasmic extensions that intersect one another (Fig. 1); the nucleus has 3 or 4 chromo centres or a prominent nucleolus.

At the end of the first week, the cells occupied the bottom of the flask almost completely, while maintaining their branched appearance (Fig. 2).

It was only in the second week that the cells become spindle-shaped, fibroblast-like, arranging themselves in bundles intersecting one another (Fig. 3) and reached confluence at the end of the second week (Fig. 4).

At this point, cells are dislodged from the flask by trypsinization and seeded on a suitably treated collagen support at a density of 1 million cells per cm$^2$.

At the beginning of the third week, under the optic microscope, chondrocytes maintain their spindle shape without extracellular matrix (Fig. 5), which starts developing towards the end of the third week; at the same time, cells become rounded or polygonal (Fig. 6).

At the beginning of the fourth week, pericellular lacunae start appearing and condense at the periphery to form capsules (Fig. 7). At the end of the fourth week, the extracellular matrix starts becoming thicker (Fig. 8).

The presence of mucopolysaccharides is highlighted by histochemical staining with Alcian-PAS (Fig. 9).
The collagen II (typical marker for hyaline cartilage) was demonstrated by means of immunohistochemical studies (Fig. 10). The other immunohistochemical studies were found to be positive for Vimentin (Fig. 11), S100, NSE and CD99. Actin, Keratin AE1, Keratin AE3, CAM 5.2, CD 57, collagen IV were found to be negative.

These results are in accordance with the data presented in studies concerning the identification of the correct immunophenotype of normal cartilaginous tissue\cite{11-34-37}.
DISCUSSION
The objective of neochondrogenesis originates from the opportunity of restoring the structural integrity and the functions of the damaged tissue by means of “regenerated tissue” that is histologically identical to the previous one, so as to be mechanically suitable for withstanding the physiological loads and reducing or avoiding arthrosic evolution of the initial lesion [13].

Hyaline cartilage is the ideal candidate for replacement since it is a non-vascular tissue [16], its trophism is ensured exclusively by the synovial liquid, has no innervation and its capacity for self-repair is extremely limited [13].

There are three main types of cartilage damage:
- matrix defect
- partial defect
- full-thickness defect

Destruction of the matrix is usually caused by a sudden trauma, such as by the dashboard in a road accident. In this case, the chondrocytes repair the matrix by increasing their synthesis activity.

Partial defects show destruction of the surface in the form of fissures which however do not extend to the subchondral bone. Immediately after the damage, the cells in the vicinity start growing but not sufficiently enough to repair the lesion completely.

The full-thickness defects are characterized by damage to the cartilage right down to the bone underneath. The defect is filled with a fibrin clot; only this type of damage allows access to the
progenitor cells from the bone marrow which can migrate into the defect; however, these cells usually form fibrocartilage in place of hyaline cartilage.

If damage to the cartilage is severe, with pain and difficulty in walking, and when the other techniques have failed, the options for treatment include removal of the articular cartilage, often followed by a prosthesis implant\(^3\). Patients with small cartilaginous lesions are currently treated by a number of methods\(^{34}\) some of which are mentioned below:

- cartilage transplant\(^{3-36}\) which may be autologous or heterologous. In the first case, cartilage removed from an area not subjected to load is used. Obviously small lesions can be treated. In the second case, transplanting fresh heterologous tissue frequently induces an immune response the frequency of which is reduced by using frozen cartilage\(^{22-33}\).
- Using periosteum or perichondrium\(^3\) in which there are undifferentiated cells capable of being transformed into chondrocytes with results that are not, however, satisfactory.
- Regeneration of cartilage\(^{36}\) by means of various methods; this involves trying to stimulate the chondrocytes present round the damaged area; unfortunately none of the substances used gave satisfactory results, especially in elderly patients. One of the methods most commonly used is penetrating the subchondral bone thereby creating a full-thickness defect to allow the mesenchymal stem cells to migrate into the defect and change into chondrocytes and/or osteocytes\(^9\). However, the cartilage formed is both fibrous and hyaline, depending on the patient\(^{10-20}\).
Other less invasive methods include, for example, treatment with laser or electrical stimulation, injection of various pharmacological substances such as growth factors or corticosteroids which, however, can lead to the formation of osteophytes\(^{[36]}\).

Transplantation of autologous chondrocytes or undifferentiated cells multiplied by means of tissue engineering to restore the missing tissue. The transplant can be done with cells in suspension or cells seeded on a suitable support\(^{[36]}\). The latter methods recently produced satisfactory, encouraging results compared to those used so far\(^{[36]}\). The clinical approach in humans mainly involved the repair of lesions in the knee, even though other sites, such as the tibio-tarsal joint, are being studied\(^{[13]}\). It is also important to identify and correct every pathological condition of the knee which is responsible for or contributes to the cartilaginous defect, before cells transplant\(^{[4]}\). After the chondrocytes were isolated and seeded in flasks, in our trial, the cells maintained their polygonal shape, long fibroblast-like, with numerous filaments, reaching confluence in 14 days. These chondrocytes are seeded and cultured on biomaterial consisting of pure collagen type I (ANTEMA\(^{®}\) SOFT, Opocrin S.p.A.), allowing their multiplication and re-expression of the original phenotype, with the production of extracellular matrix. Alcian-PAS staining made it possible to highlight the presence of mucopolysaccharides, while immunohistochemical studies showed the expression of a correct phenotype that is Vimentin positive, S100, CD99 and collagen II, the latter being a typical hyaline cartilage marker.
CONCLUSIONS

Our results show that human articular cartilage cells are capable of growing with correct hyaline phenotype in type I collagen substrate (ANTEMA®-SOFT, Opocrin S.p.A.).

We believe that these chondrocyte cultures on this biomaterial can be used for repairing cartilaginous lesions with improvement of surgical technique; the support allows adhesion of the chondrocytes to the cartilaginous lesion and a malleability that favours optimum spatial adaptation.

It is also necessary to point out the importance of suitable cyto-histological and immunohistochemical studies for evaluating the correct cell morphology and hyaline phenotype.
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Fig. 1: primary culture: chondrocytes 2 days after seeding - 400x

Fig. 2: primary culture: chondrocytes – end of 1st week - 200x

Fig. 3: primary culture: chondrocytes – beginning of 2nd week - 400x
Fig. 4: primary culture: chondrocytes at confluence 13 days after seeding - 200x

Fig. 5: H.E chondrocytes on support - beginning of 3rd week - 200x

Fig. 6: H.E. chondrocytes on support – end of 3rd week - 200x
Fig. 7: H.E. chondrocytes on support - beginning of 4th week - 200 x

Fig. 8: H.E. chondrocytes on support – end of 4th week - 400 x

Fig. 9: Alcian PAS chondrocytes on support – 4th week - 400 x
**Fig. 10**: ICH type II collagen - chondrocytes on support – 4th week - 600 x

**Fig. 11**: ICH Vimentin - chondrocytes on support - 600 x