Journal of Orthopaedics 13 (2016) 162–167

Contents lists available at ScienceDirect

Journal of Orthopaedics

journal homepage: www.elsevier.com/locate/jor

Original Article

A practical way to prepare primer human chondrocyte culture

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ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form 8 February 2016
Accepted 6 March 2016
Available online 1 April 2016

Keywords:
Chondrocyte
Osteochondral tissue
Primary cell culture

ABSTRACT

Biological cartilage repair is one of the most important targets for orthopedic surgeons currently. For this purpose, it is mandatory to know how to prepare a chondrocyte culture. In this study, our purpose was to introduce a method enabling orthopedic surgeons to practice their knowledge and skills on molecular experimental setup at cellular level, based on our experiences from previous pilot studies. Thus, we believe it will encourage orthopedic surgeons.

1. Introduction

Cartilage repair with cell therapies is a rapid developing area in regenerative medicine.[1] Recent developments in tissue engineering, pharmaceutical technology, and pharmacomolecular technology enabled researchers to use cell-based experimental methods.[3,4] Besides, cell therapies are being used to repair cartilage defects; gene therapy for an affected gene with RNA interference (siRNA/miRNA) is also used in hereditary diseases, such as Marfan syndrome, osteogenesis imperfecta, and chondrosarcoma.[5,6] In addition, cell culture methods are also currently used in biocompatibility studies of orthopedic materials, and osteo/chondro toxicity tests of pharmacological agents.

In order to use cells as an effective therapeutic for cartilage repair, they must survive and differentiate to the desired cell when they are implanted in a patient. Even if cell therapy applications have limited use in surgery at present, it could be foreseen that in a few years these studies would play an important role in many fields, including orthopedics. Therefore, it is necessary that an orthopedic surgeon is involved in these researches. For this reason, whether a researcher or a surgeon applying the treatment; an orthopedist should understand the methods to obtain and cultivating process of cells could be used in therapy.[2]

In this study, our purpose was to introduce a method enabling orthopedic surgeons to practice their knowledge and skills on molecular experimental setup, cell isolation from tissues, culture techniques, and characterization, with brief notes, based on our experiences from previous pilot studies.

2. Materials and methods

2.1. Minimum requirements for a cell culture laboratory

The first requirement, and perhaps the most important point for all cell culture laboratories, is ensuring aseptic conditions in the laboratory. In order to set up an experiment in vitro, there are some necessary equipments: an incubator which could be adjusted to 5% CO_2 and 37 °C, stereo and inverted microscope, class II biosecurity laminar airflow cabinet, a centrifuge, pH meter, vortex, heated magnetic stirrer, precision balance, sterilization system, distilled water, heated water bucket, refrigerator that could be adjusted at +4 °C, a deepfreezer that could be adjusted at −20 °C, cell counter or a Thoma Lam/Neubauer chamber, micropipettes ranging between 1 and 1000 μl, and sterile pipette tips. In addition to these equipments, necessary supplies like 5, 10, 25, and 50 mL disposable pipettes, 60–100 mm Petri dishes, glass Pasteur pipettes, autoclave resistant metal boxes, 15 and 50 mL conical centrifuge tubes, cell culture flasks, and 6-, 12-, 24- and 96-well plates are necessary. Syringe filters with 0.2 μm pore size are useful to sterilize low volume solutions.

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http://dx.doi.org/10.1016/j.jor.2016.03.008
0972-978X/

2.2. Cell culture mediums and solutions

Insulin, transferrin, and selenious acid containing premix solution (ITS), RPMI-1640 DMEM, inactivated fetal bovine serum (FBS), penicillin–streptomycin (PS), Amphotericin B, and L-glutamin are necessary to prepare a complete culture medium. Trypsin-2-[2-[bis(carboxymethyl)amino]ethyl-(carboxymethyl)amino]acetic acid (EDTA), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), and collagenase type-II enzymes are other supplements to perform primary culture.

Eagle first described the basic amino acid combinations for cell cultures in 1955 and called it "minimum Eagle's medium (MEM)". This medium is still in use with some modifications. DMEM is one of these modified mediums used especially for solid tissue cultures. RPMI-1640 contains glucose, essential for the cell nutrition, appropriate osmolarity and pH for cell viability, necessary amino acids, and vitamins for the functionality. However, it is not sufficient for the development of chondrocyte and other cells alone.[4,7] For this reason, FBS and ITS should also be added to RPMI-1640 or DMEM medium in order to proliferate healthy, high confluent cells in chondrocyte cultures.[7,8] FBS contains the essential extracellular matrix proteins for cellular adhesion and provides relevant hormones, enzymes, and growth factors for cellular proliferation. Serum proportion may differ according to cell type and applications. In standard chondrocyte cultures, 10% serum ratio is usually sufficient. L-glutamin is usually supplied with RPMI-1640 and DMEM but it is preferable to add L-glutamin to complete medium. Thus this essential amino acid preserves as carbon source. For chondrocyte primary culture, we use 1 mL L-glutamin (from 200 mM stock solution) for 100 mL medium.

Penicillin–streptomycin (PS) and Amphotericin B are used in order to prevent contamination. 1 mL of penicillin–streptomycin (10,000 U/mL) and 2.50 μg/mL of Amphotericin B are sufficient for complete medium and also for transfer medium. PS concentration could be increased in transfer medium up to 5%, but it should be noted that antibiotics have antiproliferative effects on human tissue also.[4,7,8]

ITS will stimulate cell proliferation while decreasing substantially the serum requirements for culture of many cell types. Basal media supplemented with support proliferation of chondrocyte cultures. We used a commercial ITS solution containing 5 mg/ml insulin, 5 mg/ml transferrin, and 5 μg/mL selenious acid; 5 mL of this solution is sufficient for 5 l of medium.

Collagenase type II enzyme is used to digest osteochondral tissue and obtain chondrocytes detached from extracellular matrix. Trypsin–EDTA is another enzyme used in cell culture protocols, this time for detaching chondrocytes from culture dish to passage.

PBS, a saline solution that balances intra- and extracellular osmotic pressure, supports cellular metabolism through its inorganic salt and water content. Almost every washing step can be performed in PBS.

2.3. Preparation of optimal cell culture content for chondrocytes

Transfer medium prepared with 100 mL RPMI-1640 or DMEM contained 5 mL of penicillin–streptomycin (10,000 U/mL) and 2.50 μg/mL Amphotericin B. Complete medium prepared with 100 mL RPMI-1640 or DMEM contains 1 mL of penicillin–streptomycin (10,000 U/mL) and 2.50 μg/mL Amphotericin B, 1 mL L-glutamin (200 mM), 1 mL ITS, and 10% FBS.

2.4. Surgical harvesting and transfer of osteochondral tissue

Tissues from lateral and medial femoral condyles, and tibial plateau, are routinely removed during total knee arthroplasty (Fig. 1). After surgery, leftover osteochondral tissue is immediately transferred to cell culture laboratory in transfer medium under sterile conditions. Keeping tissue at 4 °C with ice packs prevents tissue from cell death. Attention to aseptic conditions, keeping tissue in cold, and immediate transfer are some of the key points for obtaining healthy primary cultures.

2.5. Preparation of primary chondrocyte cultures

Local ethics committee approval should be ready for the relevant animal or human tissue to be studied. If a human tissue is to be studied, an informed consent form is also necessary in addition to local ethics committee approval.

A common method to obtain single cell suspensions from primary tissue is enzymatic digestion. Exposing the cells to collagenase enzyme preserves maximum viability. The following procedures disaggregate whole tissue to obtain a high yield of viable cells. Note that all the following procedures are performed in a laminar flow cabinet.

Resected tissues from human or animal osteochondral tissue were transferred to cell culture laboratory and transferred to a Petri dish and smashed into small pieces, approximately 0.25 cm^3, either with scalpel or rongeur. To prevent from drying, the tissue is always kept in transfer medium or PBS. The tissue is transferred to 50 mL conical tubes and centrifuged at 1200 rpm for 10 min. The supernatant is discarded. To perform enzymatic digestion,

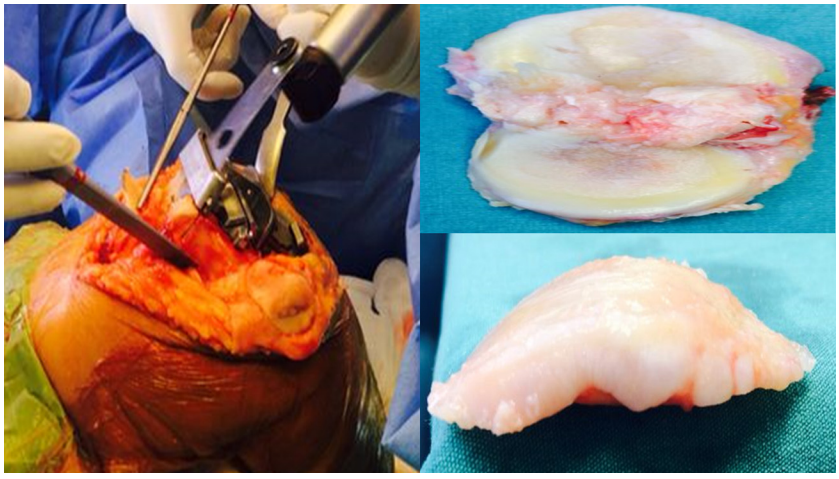

Fig. 1. Surgically excised osteochondral tissues.

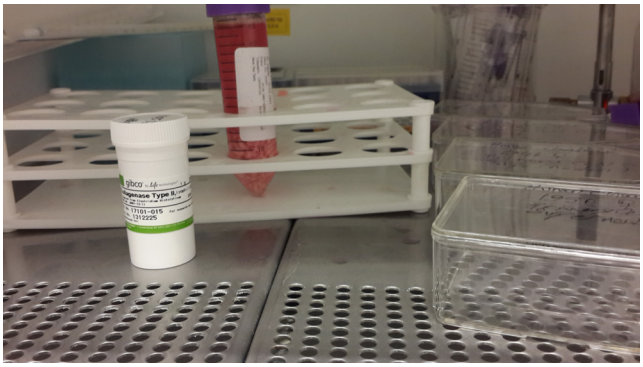

Fig. 2. Adding collagenase to mechanically disintegration tissues.

200 units/mL collagenase type II enzyme mixture, dissolved in complete medium, was used. Collagenase type II solution is added, mixed gently, and transferred to a Petri dish and incubated tissue for 16 h in a CO_2 incubator (Fig. 2).

Afterwards, tissue samples were centrifuged at 4 °C at 1200 rpm for 10 min to discard collagenase. Sedimented cartilage and bone tissue pellets were resuspended in fresh culture medium (DMEM or RPMI-1640), and transferred to flasks to obtain primary cultures (Fig. 3).

2.6. Inverted microscope

Inverted microscope is used to determine cell morphology. Attachment, viability, and proliferation could be monitored with 4×, 10×, 20×, and 40× optical magnification. With a charge-coupled device (CCD) camera attached to inverted microscope microphotos could be obtained and documented (Fig. 4).

2.7. Passaging the chondrocytes

Cells proliferate in a logarithmic scale in in vitro conditions and cultures became confluent according to doubling times of cells where they originated from. Too confluent cultures even cell lines because of contact inhibition proliferation terminates. Passaging cultures stimulate cell division and terminate stationary phase. Every laboratory or every study has its own approach to passage cells. Usage of ice cold PBS could easily detach cell lines from culture vessel but usually primary cultures are more sensitive to cold and more fragile to mechanical effects. Usage of scraper is

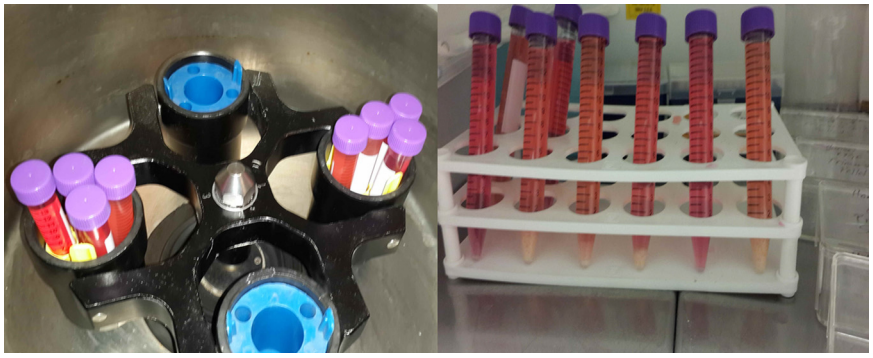

Fig. 3. View of pellet after cooled centrifuge.

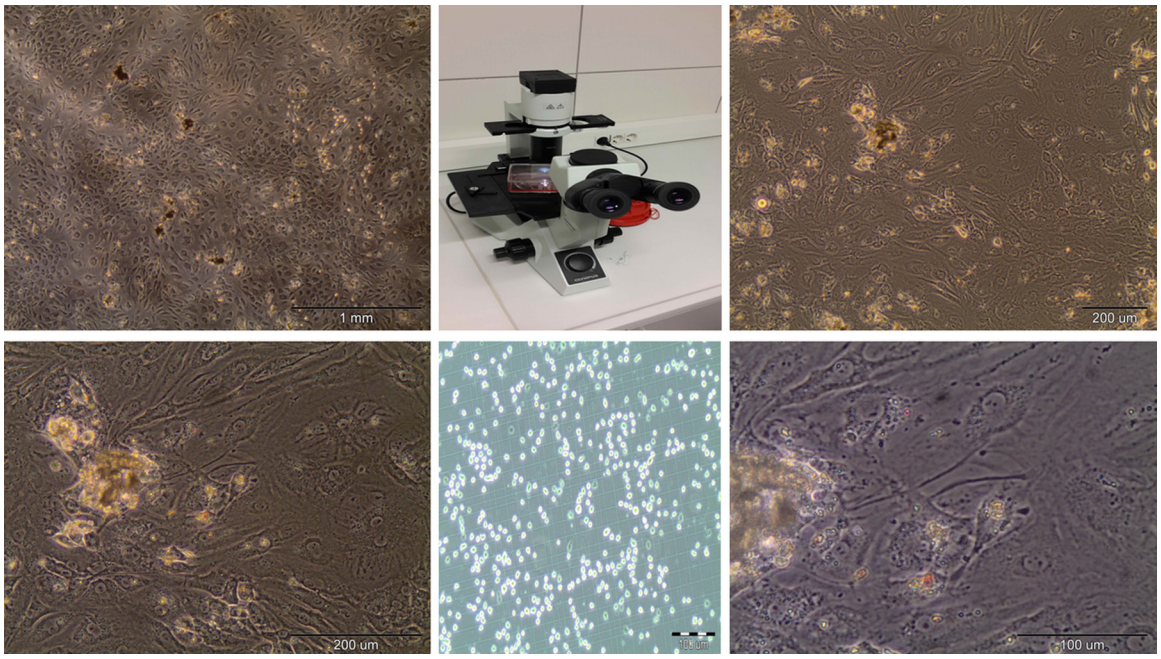

Fig. 4. Healthy and proliferating cells with 90% confluence and cell count on Thoma counting chamber with trypan blue.

Table 1
Amount of trypsine to be added.

Size of Petri dish	Trypsine (0.25% EDTA)	The amount of complete medium to inhibit proteolytic reaction containing 10% FBS
100 mm	2 mL	4 mL
60 mm	1 mL	2 mL
35 mm	0.5 mL	1 mL

another technique to collect cells from vessel but as mentioned before you can damage your culture mechanically.

We prefer to use trypsin–EDTA as a proteolytic enzyme to passage our chondrocyte cultures. FBS in complete medium has factors that can inhibit trypsin's activity; because of this, before applying trypsin solution, cultures are washed with prewarmed PBS. The key point of using trypsin is not to expose cells too long to trypsin and to terminate the enzyme activity with complete medium when cells detached from surface eventually.

Cell detachment can be easily monitored with an inverted microscope (Table 1). Afterwards, the content is transferred into falcon tubes and centrifuged at 1200 rpm for five minutes. Following centrifugation, supernatant is removed and pellet should be feeded with fresh complete medium. Trypsin should be kept at −20 °C in order to keep it active. Freeze–thaw cycle inhibits the enzyme activity, so aliquatting is recommended.

2.8. Scanning electron microscope (SEM)

To prepare cell culture for SEM, we use glutaraldehyde and cacodylate buffer for fixation of the chondrocytes. We add a 2.5% glutaraldehyde solution, composed of 97.5 mL of cacodylate buffer and 2.5 mL of glutaraldehyde, to the culture vessel to cover the cells and left for incubation at room temperature for 130 min. Following fixation, cells are washed with only cacodylate buffer three times. SEM analysis could be done instantly or cells can be kept in dark at 4 °C up to one week preventing from drying with cacodylate buffer. If environmental scanning electron microscope (ESEM) is to be used instead of scanning electron microscope, these samples could be viewed after supernatant is removed without the need for coating with silver or gold (Fig. 5).

ESEM analysis assesses the surface topography and composition of the cells. We use a device with a lifting system and the ability to transfer the electron beam in a high vacuum. ESEM analysis enables us to obtain images of the extracellular matrix, as well as images of characteristic cellular structures.

2.9. Indicator of terminal phenotypic identification

In literature, authors mentioned that molecular identification of Cbfa-I, type II and IX collagen, and aggrecan molecules would be enough for phenotypic identification of chondrocytes.[10] In addition to that, it is also mentioned, that if desired on cellular level, proteoglycan, type II, and IX collagen-rich matrix content could be also investigated.[8–10] Recently, identification is available by examining aggrecan, CD44, CD151, collagen II and IV, DSPG3, and sulfated proteoglycan markers.[11] It is also possible to evaluate chondrocytes with commercial kits, which is called human chondrocyte characterization, and uses enzyme linked immunosorbent analysis (ELISA) or human flow cytometric analysis. These relevant commercial kits work by assessing the proportion of SOX-9 protein of chondrocytes in the primary culture.

Using these kits rules out the need for preparation of cell lysates and thus provides an ease-of-use, particularly in drug discovery and cell chondrotoxicity in addition to facilitating an experiment and providing an effective experiment. Moreover, it should be kept in mind that they could also be used to evaluate the inhibitor effect of combinations and ligands.[12]

2.10. Immunoflow cytometry

Immunoflow cytometry identifies monoclonal antibodies expressed by the cells, which could be used for clinical diagnosing and research. Cells are marked in a suspended format and are detected in different photodetectors using different colors, such as propidium iodide (PI), ethidium, Texas Red, Fluorescein isothiocynate (FITC), and phycoerythrin (PE). However, if the analysis is made with light colors, false positive or negative results may occur.

In our laboratory, we use commercial ELISA-based kits or flow cytometry to characterize our primary chondrocyte cultures. To perform flow cytometry, firstly cells are examined by inverted microscope and the cells proved to have adhesion to the surface and have sufficient confluency. Then chondrocytes are detached from culture vessel using trypsin–EDTA and transferred to a falcon tube. After centrifugation at 4 °C in 1200 rpm for 5 min, pellet was resuspended with complete medium and the cells are counted with

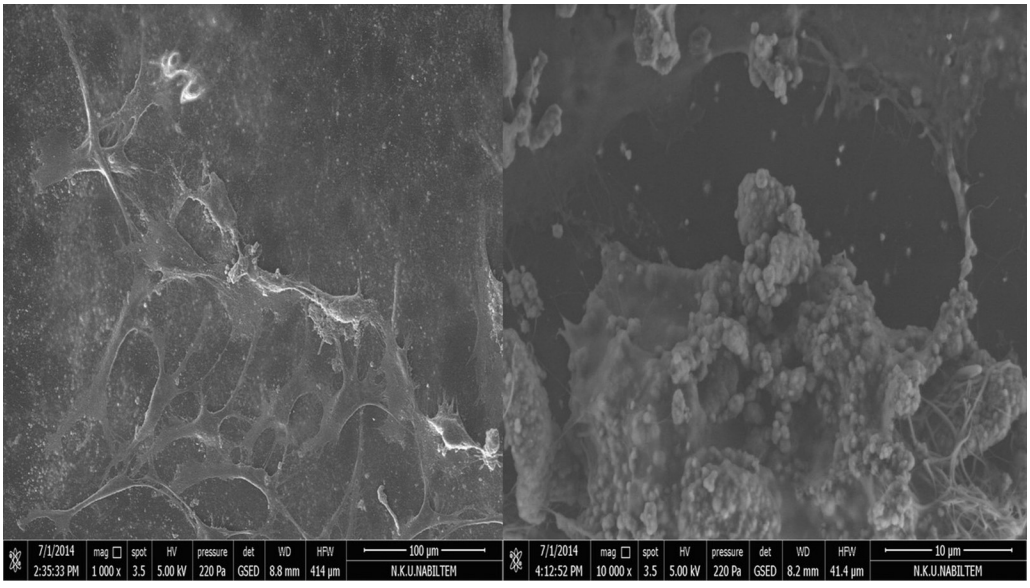

Fig. 5. Morphological evaluations of surface.

Table 2
Proper cell number and complete medium volumes for chondrocyte primary cultures.

Culture vessel	Cell number	Maximum complete medium volume
Petri dish (100 mm)	5×10^6	10 mL
Petri dish (60 mm)	2×10^6	5 mL
Petri dish (35 mm)	1×10^6	3 mL
Well plate (24)	125×10^3/1 well	300 μl

Neubauer chamber. Suspension of cells containing desired number of cells are incubated with fluorochrome-conjugated monoclonal antibodies, such as FITC–PE at RT for 50 min. PBS prepared with 0.1% sodium azide is added to this cell suspension and centrifuged for 5 min. Supernatant is removed, resuspended cells are analyzed with flow cytometry, and analyses are evaluated by the software.

2.11. The counting and even distribution of the cells into samples

Neubauer chamber or hemocytometer is routinely used for cell counting. Neubauer chamber or hemocytometer slide surfaces contain 1 mm thick striped squares and smaller squares inside these. Simply suspended, cell sample spread to slide and is counted. For detailed information how to count cells with Neubauer chamber or hemocytometer. In order to detect viable cell number, trypan blue dye is used. The reason for using the viability color trypan blue during cell counting is that it only stains dead cells entering through cell membrane and this provides quantitative analysis for cell counting. This process also allows counting dead and alive cells and analyzing dead–alive cell ratio (Fig. 4).

Since trypan blue cannot enter into live cells, they look smaller, refractile, and more rounded when compared with dead cells. Dead cells look bigger and in Prussian blue like color. Cell numbers and complete medium volumes we recommend for chondrocyte cultures are given in Table 2.

2.12. Viability, proliferation, and toxicity analyses with commercial ELISA kit

Cell viability and proliferation could be quantified through comparison of the absorbance values of control groups with experiment group using an ELISA-based cell viability kit (it estimates a percentage based on evaluation of metabolic activity with microplates). Absorption rates of the control group could be considered 100% for cell viability.[4,7,8]

Commercial kits used in this analysis, such as MTS or MTT, contain a colorimetric substrate, which is a tetrazolium salt. In live cells, with the activity of mitochondrial dehydrogenase enzymes, tetrazolium salt reduced into insoluble formazan crystals, which has color between thiazole blue and purple, detectable with spectrophotography. However, dead cells could not form these formazan crystals. Every commercial kit has its own protocol but proliferation and proliferation inhibition could be found with the following formulas, respectively: proliferation = (Test OD/Control OD) × 100 and proliferation inhibition = 1 − proliferation. Thus, live cells with active mitochondria could be easily quantitated.[4,7,8]

3. Discussion

Developments in regenerative medicine have already been effective in orthopedics, particularly on chondral regeneration. The ultimate goal has been biological repair of the chondral damage.[8,13] Parallel to the advances in technology, researchers are focusing on this field and also culturing chondrocytes in vitro.

Recently, it is desired to produce healthy cells, which have desired morphology, and are alive and productive.[7] It is obvious that primary cultures have characteristics of the original tissue, and since they are heterogeneous, they are superior to single cell line cells. In addition to that, it would be more convenient to use heterogeneous cell cultures instead of single line cells in laboratory in terms of side effects after they are implanted to host.[6,7] For this reason, different methods have been used to isolate cells.[5,12–14]

Isolating cells from solid tissue that is surgically excised is the first key point to obtain a healthy chondrocyte culture. The researcher needs to prevent cells from damage, generated from mechanic or enzymatic applications.[6,15] Cells should dissociate from surrounding extracellular matrix as well as from each other.[16] For this purpose, general proteases like trypsin or tissue-specific enzymes like collagenases are used. At the end of this process, successful cell suspensions with heterogeneous content are obtained.[3,4,6,16–18]

Cells with a high nuclear cytoplasmic ratio could be distinguished from other cells by gradient centrifugation. Specific cell markers, cell membrane receptors, or signal molecules could also be used for distinguishing. Both protein groups have different affinity to a specific cell. It is also possible to distinguish cells by florescent marking of a signal molecule or antibody.[15–20]

In our studies, we use type II collagenase to isolate osteochondrocytes from tissue, which is the most appropriate way.

Following the isolation process of the cells, the researcher needs to identify how to feed cells also for each cell type.[6,21] Cells and feeding xenocultures or pharmaceuticals should be approved by FDA and should be chosen from materials that were tested to be effective against infectious agents.[21] More importantly, biochemicals to be used in cell culture mediums should be tested in terms of endotoxins.[21] In some studies, human-derived cell layers are used in feeding instead of mouse embryonic fibroblasts in order to prevent inter-species xenotypic contamination or pathogen contamination.[22] However, in this condition, it is difficult to differentiate cells in study group and the feeding layer. For this reason, it should be noted that the sign "endotoxin and cell culture tested" is available. Moreover, it is also important that the researcher uses substrates, such as fibronectin and laminin or simple hydrogel systems, in order to attach cells on Petri dish, flask, or well surfaces in layers, which do not contain nutrient.[23]

If the above-described system or substrates are not used, chondrocytes will not adhere to the surface of the flask and they will be thrown out with supernatant during exchange of complete medium. As a result of this, cell will not have sufficient number and viability. This will increase the necessary time to reach the desired number of cells, and expensive materials will be used, which will not be cost effective.

Researches reporting the use of TGF in cartilage regeneration as in studies of Chua et al. or Beane et al. are available in literature.[8,24] However, it should be noted that TGF-β1 has the late side effect, which causes transformation into fibrous cartilage instead of hyaline cartilage.[4]

Goldring described a protocol in which it is possible to use Dulbecco's modified Eagle's medium (DMEM) and ITS together for chondrocyte proliferation in a study titled as "Human Chondrocyte Cultures as Models of Cartilage-Specific Gene Regulation".[25]

In our studies, we use DMEM and RPMI-1640 supplemented with ITS. We have obtained similar proliferation rates with both mediums. Aung et al. reported that they changed the cell culture medium content once every 7 days. In addition to that, they also reported that they used DMEM and ITS together in order to enhance chondrogenic activity and cell proliferation.[26]

We recommend changing complete medium in every 2 days. Cell metabolism causes change in osmolarity and pH of the medium and eventually cells will die. Although MEM or DMEM are the most used mediums in somatic cell cultures, the importance of the mediums, which are compatible with ITS, is reported in

chondrotoxicity studies.[6] Moreover, some studies also suggested that chondrocytes could proliferate with transforming growth factor (TGF)-β3 and ascorbic acid together or TGF-β1 alone instead of using ITS premix solution.[4]

Kuske et al.[27] reported in a study that insulin-like growth factor-1, bone morphogenic protein (BMP)-2, ascorbic acid, DMEM, and MEM should be used during the differentiation of embryonic stem cells into chondrocytes.

It should not be forgotten that BMPs will induce osteogenic differentiation more than chondrogenic differentiation. For several reasons including that, it is advised to use RPMI-1640, FBS, PS, and ITS in complete medium for chondrocyte cultures.

During setting up of the primary chondrocyte culture, it should be remembered that no matter which method is used, it is important to note the origin of the isolated tissue and the age of the donor.[17,18]

Cells will proliferate in colonies starting from the sixth day. The bright border with a dark center of a round shape is indicative for viability of colonization. Smooth cells surrounded with fibroblast-like cells is suggestive of the differentiation. If not desired otherwise, overgrowth and over passaging of the cell colonies should not be allowed. Maximum number of passaging of chondrocyte cell cultures should not exceed.[4,6]

It is known that after passaging several times, immunophenotypic characteristics of the cells are investigated over expanded cells rather than primary cell cultures. There is no indicator for chondrocytes as CD44 is for osteocytes, which is a receptor for binders, such as hyaluronan and osteopontin.[28–30]

Cells stained with different colors and density of florescence could be differentiated using flow cytometric methods or in vivo and/or in vitro fluorescent-activated cell sorting (FACS) methods or ex vivo immunofluorescent staining.[25] Although it is possible to differentiate with these methods, it is important to confirm the results with gene expression analysis.[31] Western blotting could be used after either mass spectrophotometry, which is used in identification of protein chain reaction, microarray, proteomic profiles of cell gene expression, or sodium dodecyl sulphate polyacrylamide gel electrophoresis, which is used for protein quantification.[4,7] In addition to this, new array based techniques, such as new generation DNA sequencing, could also be used in identification of post-translational modification or gene expression.[31]

4. Conclusion

Biological cartilage repair is one of the most important targets for orthopedic surgeons currently. For a research or application in this regard, obtaining the appropriate cells with tissue culture methods is the first step. Orthopedists should learn about the tissue and cell culture methods, as practitioners, and even taking part in multidiscipline work will improve the quality of research to be done. Especially for the studies involving molecular experimental series, good planning is very important. In the planning stage of a project, knowledge of the clinician takes a important place.

Conflicts of interest

All authors have none to declare.

Funding statement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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