MERIT RESEARCH JOURNALS
www.meritresearchjournals.org

Merit Research Journal of Medicine and Medical Sciences (ISSN: 2354-323X) Vol. 6(3) pp. 100-110, March, 2018
Available online http://www.meritresearchjournals.org/mms/index.htm

Original Research Article

Which pharmacological agents may be used to establish healthy, proliferating human primer nucleus pulposus / annulus fibrosus cell cultures? Systematic evaluation of our experience in the light of literature

Numan KARAARSLAN[1*], Ibrahim YILMAZ[2], Feride Sinem AKGUN[3], Tezcan CALISKAN[1], Abdullah Talha SIMSEK[4], Necati KAPLAN[5], Yasin EMRE KAYA[6], Duygu YASAR SIRIN[7], Hanefi OZBEK[8] and Ozkan ATES[9]

[1]Assist. Prof. M.D., Namik Kemal University School of Medicine, Department of Neurosurgery, 59100, Tekirdag, Turkey.

[2]Medical Pharmacologist, Pharmacist, Istanbul Medipol University School of Medicine, Department of Medical Pharmacology, 34810, Istanbul, Turkey.

[3]Assist. Prof. M.D., Istanbul Maltepe University School of Medicine, Department of Emergency Medicine, 34857, Istanbul, Turkey.

[4]Sepecialist M.D., Republic of Turkey, Ministry of Health, State Hospital, Department of Neurosurgery, 36200, Kars, Turkey.

[5]Assist. Prof. M.D.; Istanbul Rumeli University, Corlu Reyap Hospital, Department of Neurosurgery, 59680, Tekirdag, Turkey.

[6]Assist. Prof. M.D., Abant Izzet Baysal Univesity School of Medicine, Department of Orthopaedic and Traumatology, 13100, Bolu, Turkey.

[7]Assist. Prof. Ph.D., Namik Kemal University Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, 59100, Tekirdag, Turkey.

[8]Prof. M.D., Ph.D., Istanbul Medipol University School of Medicine, Department of Medical Pharmacology, 34810, Istanbul, Turkey.

[9]Prof. M.D., Istanbul Esenyurt University, Esencan Hospital, Department of Neurosurgery, 34510, Istanbul, Turkey.

***Corresponding Author's É-mail: numikara@yahoo.com**
Phone: 282- 2507140
Fax number: 282-2509950

Abstract

Intervertebral disc tissue is damaged and loses its normal physiological functions due to age-related degeneration, vigorous physical activity and trauma in all age groups and at any stage of life. Here we aim to present how to prepare human primary cell cultures effectively from intact intervertebral disc tissues obtained from the patients undergoing operations for spinal trauma and, from degenerated disc tissues obtained from patients with a lumbar herniated. In addition, it is aimed to explain bio/pharmaco-molecular methods used in their analyses at a molecular level in line with the current literature. Articles included in Pubmed between June 1988 and January 31, 2018, were searched with the key words "nucleus pulposus cell proliferation" and/or "annulus fibrosus" without language restriction. Data of descriptive statistics were analyzed using mean ± standard deviation or frequency (%) and demonstrated using Microsoft Office 2010 and Excel. In addition, it was explained how to obtain human primary cell culture from the tissues taken from two different localizations of the single intervertebral disc. A total of 419 articles were found to be potentially relevant, as a result of first search. In this literature review, cell cultures, which could possibly be the main focus of cell therapies in the field of neurosurgery in the near future, as well as current data were discussed from a molecular point of view.

Keywords: Annulus fibrosus, Acridine orange, Propidium iodide staining, differentiation, nucleus pulposus, primary cell culture technique, proliferation.

INTRODUCTION

Thanks to the advances in pharmaceutical technology and pharmaco-molecular branches, experiments carried out in the laboratory have become indispensable in search of new therapeutic modalities developed to prevent many diseases, or in the analysis of cytotoxicity of newly introduced drugs or bio-implants (Akyuva et al., 2017; Isyar et al., 2015). Laboratory production of cells have gained wide popularity, especially in research on the treatment of incurable diseases by regenerative or reparative methods (Akyuva et al., 2017; Isyar et al., 2015; Komur et al., 2017; Topuk et al., 2017). Accordingly, to replace or repair the biological activities of damaged cells and tissues, cell culturing techniques have begun to be used in neurosurgical research for personalized cellular therapy methods (Gobbel et al., 2003; Wiet et al., 2017; Lin et al., 2017).

During the application of cell culture techniques, independently of the type of cells used, cellular characteristics may differ depending on the effect of stimulant pharmacochemical agents added to their environment (Akyuva et al., 2017; Gökçe et al., 2014; Isyar et al., 2016; Karaarslan et al., 2018). Different substances, such as insulin, transferrin, selenic acid, amino acid, carbohydrate, lipid, vitamin and/orleptin were added to the cell culture environment to increase the differentiation potential and to support the growth and protection of the cells (Akyuva et al., 2017; Gökçe et al., 2014; Isyar et al., 2016; Karaarslan et al., 2018).

The aim of molecular-based experimental studies is to find the most effective method as well as to reduce the costly methodologies that bring an excessive financial burden on the health economies. The importance of ensuring effective and healthy cell proliferation using a less costly experimental setup is emphasized in literature (Isyar et al., 2016).

In this study, we assessed the question which pharmacological, biological or pharmaceutical agents may be used to prepare more consistent primary cultures of both human nucleus pulposus (NP) cells (NPCs) and annulus fibrosus (AF) cells (AFCs). The goal of this review was not to provide information to neurosurgeons on how to prepare primary human disc cell cultures from intervertebral disc tissues by isolating NPCs/AFCs but to encourage them on how to systematically characterize the disc tissue cells from in-vitro cellular experimental setups obtained from surgically resected tissues.

MATERIALS AND METHODS

Materials

Medical Consumables and Pharmaceuticals

Laboratory equipment required includes an incubator, which can be set to a temperature of 37°Cand 5% CO_2, stereo and inverted light microscopes, pH meter, vortex, heated magnetic stirrer, precision scale, laminar air flow cabinet with at least class II bio-safety, heated water bath, refrigerated centrifuge, cell counting chamber, basic equipment such as various sterile pipettes ranging from 1-1000 µL.

Falcon tubes 15 and 50 mL are also needed. In addition, glass or porcelain pharmacist muller, scalpel tip and scalpel handle are also needed to mechanically break down the tissues. The necks of the flasks to be used should be curved like a swan. It is also important that flask caps have pores that allow ambient gases in the incubator to enter and exit the flask. Well plates that have 4, 6, 24 and 96 wells will make research easier in the cultivation steps, especially during the trypsinization phase.

Apart from the equipment indicated above, the following is also needed: Premix solution consisting of insulin, transferrin and selenic acid (ITS), Modified Eagles's medium (DMEM), Dulbecco Modified Eagles's medium (DMEM), RPMI-1640 medium, inactivated fetal bovine serum (FBS), penicillin-streptomycin (PS), trypsin-ethylene-diamine-tetraacetic acid (EDTA) solutions, phosphate-buffered saline (PBS), Hanks' Balanced Salt Solution, dimethyl sulfoxide (DMSO), and collagenase type I and type II enzymes.

In addition, filters with pores diameters of 0.2 µm are required for filtration of PBS, distilled water or FBS.

Methods

Search Strategy

Studies, conducted between June 1, 1988 and January 31, 2018, in US National Library of Medicine National Institutes of Health (NLM) (PubMed), Embase, OVID, Cochrane Library electronic databases were scanned using terms "OR", "AND" without language and country restrictions. On-line literature searches were carried out using the key words "nucleus pulposus cell proliferation", "annulus fibrosus", individually or in combinations (Figure 1).

The study carried out by Lijmer et al. (1999) was used to determine the level of evidence of the studies. Subsequently, the data obtained were checked using Transparent Reporting of the Systematic Review (PRISMA). Appropriate studies, with a higher-evidence value, involving cell cultures of intervertebral disc tissue were investigated during the research. The reference lists were also evaluated in terms of the availability of appropriate articles. Unpublished studies found in electronic databases, such as Researchgate were not included in the evaluation. Unpublished literature,

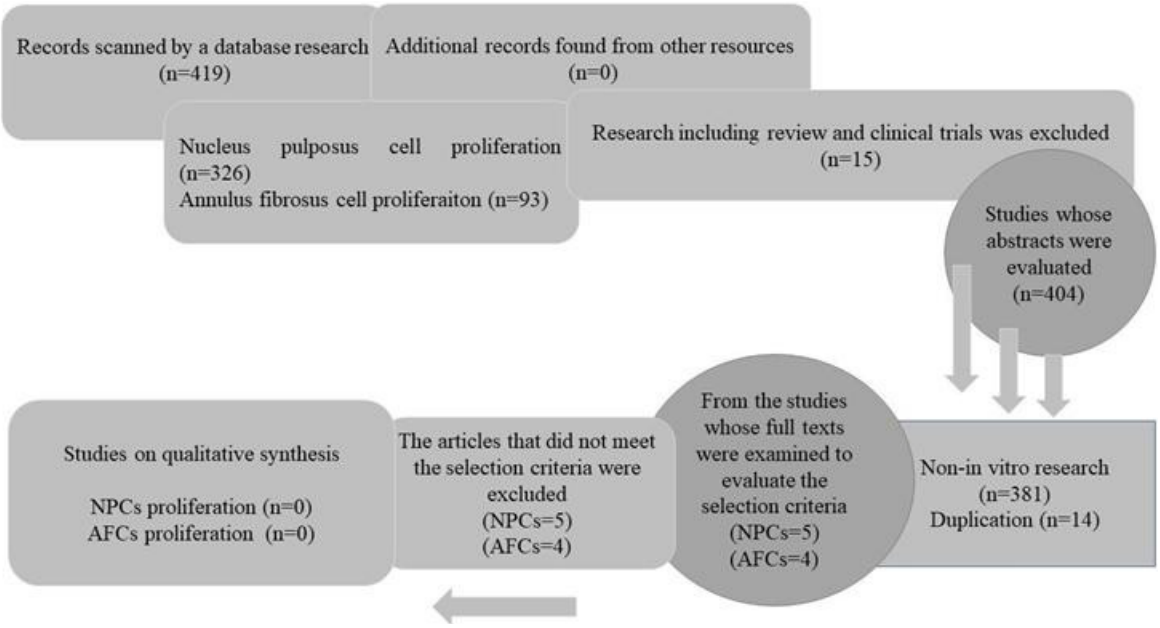

Figure 1. Article scanning process in literature.

including articles, comments, letters, editorials, protocols, guides, meta-analyses, and collections, were not included. The citation index of the articles was determined using the *Web of Science* and *Scopus database.* References and citations of all articles were examined to avoid possible repetitions.

Exclusion criteria

Non-controlled, non-blind and non-comparative studies regarding "Nucleus pulposus cell proliferation" and "Annulus fibrosus" were not included in the scope of the research.

Accumulation and Evaluation of Data

The authors independently selected the included studies. The risk of selection bias, which might be caused by potentially masking, was also investigated. All studies were examined by seven authors (NK, FSA, IY, TC, NK, YEK, and DYS*)* to ensure accuracy. In cases where there was disagreement between at least two authors and consensus was not reached, the topic discussed by the authors with highest research experience (HO and OA) was examined.

Statistical Analyses

Review Manager Software Program in Windows XP was used to evaluate data obtained from appropriate studies. Heterogeneity was measured using the Cochrane Q test. Descriptive statistical evaluations were performed using the Microsoft Excel program (2013 version). Descriptive statistics were expressed as mean ± standard deviation or frequency (%).

Preparation of Fresh Medium

Cell Culture Media Content (CCMC), needs to be prepared freshly for each use. The flasks are placed into the laminar flow cabinet, and their caps are opened. 50 mL of FBS is added to 500mL of 1XRPMI-1640, MEM or DMEM medium and passed through a filtre. Subsequently, 5 mL of PS and 15 mL of ITS solution is added. Cells in culture must be daily monitored with an inverted light microscope and can be kept for a maximum of only four days without changing CCMC. Later on, cells in the flasks can be fed once every two days until they are taken into experiment. In this way, metabolic wastes emerging from the food and gas exchanges of the cells are cleared from the flasks.

Selection and exclusion criteria for tissue culture studies

Firstly, approval from the ethics committee for the establishment of primary cell cultures from tissues and informed consent should be obtained, in accordance with the Declaration of Helsinki. The tissues resected for the use in the study are kept sterile in the operating room and transferred (4° C) to the Falcon tubes 50 mL containing

medium. A barcode, which carries the patient data, should be used to label the resected tissue.

Patients with a poor nutritional status, human immunodeficiency virus, bone marrow hypoplasia, leukopenia, thrombocytopenia, anaemia, and the cases with organ failure, such as kidney and/or liver failure should not be included in the study. In addition, tissues from pregnant and breastfeeding mothers, or patients with a history of alcohol addiction and/or known drug abuse, or egg protein allergy should be excluded (Akyuva et al., 2017). It is also important to exclude patients using drugs that affect the enzyme levels in the body or that interact with the cytochrome P450 (CYP2A6) enzyme(Akyuva et al., 2017; Karaarslan et al., 2018). Tissues of the patients that use disease-modifying anti-rheumatic drugs, or biological therapy agents, such as rituximab, etanercept, adalimumab, abatacept should be excluded from the study (Dogan et al., 2016; Gokce et al., 2012; Gumustas et al., 2016; Gumustas et al., 2017; Guzelant et al., 2017; Isyar et al., 2015; Isyar et al., 2016; Karaarslan et al., 2018; Komur et al., 2017; Oznam et al., 2017; Topuk et al., 2017). Exclusion criteria should be used to prevent confounding data during research.

To obtain degenerated intervertebral disc tissue, detailed physical and neurological examinations of cases with waist and/or leg pain should be performed. Surgical intervention should be planned in patients, who have neurologic motor and/or sensory deficits, reflex deficits, sphincter defects and who still have complaints of pain despite long-term medical treatments, diagnosed with herniated NP after imaging examinations and electrophysiological tests. During surgical intervention, degenerated intervertebral disc tissue can be obtained.

To prepare the intact intervertebral disc tissue, non-degenerated disc tissue can be obtained intraoperatively from the patients who applied to the hospital with spinal trauma and traumatic disc herniation.

Resection of Tissues

All patients are placed in the prone position under endotracheal general anaesthesia, and the surgical level is confirmed by C-arm scopy. Surgical field antisepsis is provided with 10% polyvinyl pyrrolidine iodine solution and covered in a sterile manner. To prepare degenerated NPCs/ AFCs cultures, a para-median skin incision, according to the localization of herniationis made, and a lumbar discectomy. The lumber superficial fascia is incised. In the patients with median, paramedian, and foraminal disc herniation, para-vertebral muscles are dissected subperiosteally after performing a fascia incision through a median approach. Hemi partial laminectomy is then performed microscopically. Finally, microdiscectomy is performed following the flavectomy and degenerated NP tissue can be obtained.

In patients with a far lateral disc herniation, superior and inferior facet joints of the pars interarticularis and herniation level are identified transmuscularly after performing a fascia incision through median approach. Discectomy is performed by reaching the tissue of the degenerated herniated NP through afar lateral approach, and degenerated NP tissue can be obtained.

Preparation of intact NPH/AFH cultures; in patients with secondary instability and traumatic disc herniation after spinal trauma, the skin is penetrated and a median skin incision is made. Para-vertebral muscles are then dissected subperiosteally after bilaterally incising the lumbar superficial fascia. If spinal cord compression due to traumatic disc herniation is detected, laminectomy and discectomy should be performed at this level. Superior and inferior total discectomy are performed in conjunction with corpectomy, in cases where the cord pressure has stemmed from the traumatic vertebral fracture. NP tissues obtained from both surgical methods can be used to prepare intact NP/AF cell culture.

Sterile tissue sections are place in the Falcon tubes containing DMEM with 5% PS and transferred to the laboratory at 4 °C.

Preparation of primary cultures

The tissues are taken into the laminar flow cabinet and transferred to the first of five sterile petri dishes. They are subsequently washed sequentially with either sterile PBS, pH 7.4, or isotonic sodium chloride solution 0.9% (sterile) to clean the tissue from red blood cells. The tissues are then broken down mechanically by using pre-sterilized porcelain or glass mullers in the laminar cabinet. Tissue samples, are shredded to the extent of minced appearance, and/or enzymatically shredded with 475 µg *Clostridium Histolyticum*-derived collagenase type I and type II enzymes dissolved in HBSS. To accomplish this, tissue samples subjected to enzyme addition are incubated at 37.4°C and 5% CO_2. These tissues are then incubated overnight and centrifuged at 4°C and 1.200 rpm consecutively twice for 10 minutes (Figure 2). The cell suspensions are transferred to flasks or petri dishes and kept in the incubator for at least 72 hours. Adherent cells are used when they reach about 90% confluency.

Cells multiply logarithmically and when they occupy almost the entire surface area they are in, they gradually become unable to proliferate and require cell passaging. First, cells can be removed from the adherent surface using special sterile cell scrapers without using any chemicals. Alternatively, cells can be passaged using proteolytic enzymatic reactions, such as collagenases, trypsin solution or EDTA alone or in combination. Since cell scrapers may affect cell integrity, the use of a trypsin-EDTA mixture which can be inhibited by the addition of CCMC would be more appropriate for the passage of

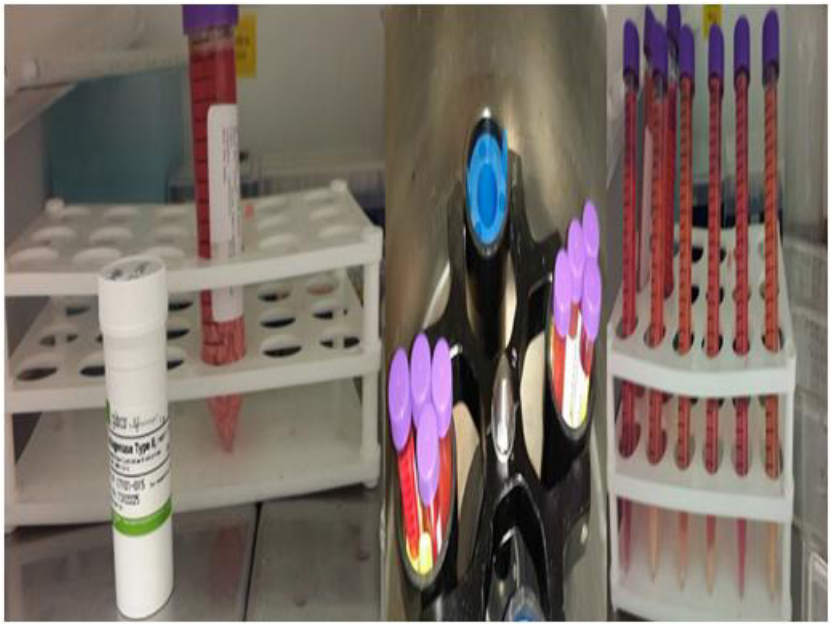

Figure 2. Re-suspended image of cell pellets remaining in the floor of the tubes in the form of a yellowish -whitish thin layer, with CCMC prepared with DMEM.

NPCs/AFCs cultures. Before trypsinization, the CCMC in the medium should be removed, because the serum containing CCMC may contain trypsin inhibitors and the container should be washed with Ca^{2+} and Mg^{2+} free PBS. Thereafter, the trypsin solution is added on to the flask. Using an inverted light microscope, detachment of the adherent cells from the surface can be monitored. The proteolyticreaction should be stopped by adding twice as much CCMC the amount of added trypsin, since the trypsin may damage the cell membranes after separating the cells from the surface.

Viability Assays

In primary cell cultures obtained from humans, cells should be seeded in cell culture dishes of diverse sizes and characteristics according to the analysis, under the inverted light microscope, and counted with the help of a hemocytometer in the presence of Trypan blue (Akyuva et al., 2017; Dogan et al., 2016; Gokce et al., 2012; Gumustas et al., 2016; Gumustas et al., 2017; Guzelant et al., 2017; Isyar et al., 2015; Isyar et al., 2016; Karaarslan et al., 2018; Komur et al., 2017; Oznam et al., 2017; Topuk et al., 2017).

Single cells are gently resuspended with the aid of a micropipette. To calculate cell numbers, 10 µL of the diluted cell suspension in 1 mL of culture medium is added to the Eppendorf tube and mixed with the 90 µL of Trypan blue dye. This mixture is placed on the hemocytometer, such as a Thoma slide and live cells found in at least 5 sections in the slide are counted. The number found is multiplied by the amount of dilution and 50,000. In this way, it is calculated how many million cells are present in 1 mL of medium. After the number to be seeded is determined, the cells are seeded in the flasks, well plates or petri dishes (Figure 3).

Confluent cells should then be replated into a number of subgroups. All plates should be kept in an incubator after adding freshly prepared media for at least 24 hours at most 72 hours at 37,4°C and 5% CO_2. Depending on the purpose of the research and the experimental setup, these measurement days can be further extended by certain periods.

For evaluation of the cell cultures; at least one of the following should be applied: inverted light microscopy, immune flow cytometry, MTT cell viability, toxicity and proliferation assays and/or environmental scanning electron microscopy (ESEM). For assessment of gene expression of markers, such as NPCs-specific chondroadherin, hypoxia-induced factor-1α, extracellular matrix-specific glycosaminoglycans, various collagen or matrix metalloproteinases or inhibitors thereof, or various interleukin types involved in inflammation quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) can be performed (Akyuva et al., 2017). In addition, array-based techniques, such as next-generation sequence analysis can be used as the most up-to-date technique for determining post-translational modifications other than gene expression (Karaarslan et al., 2018).

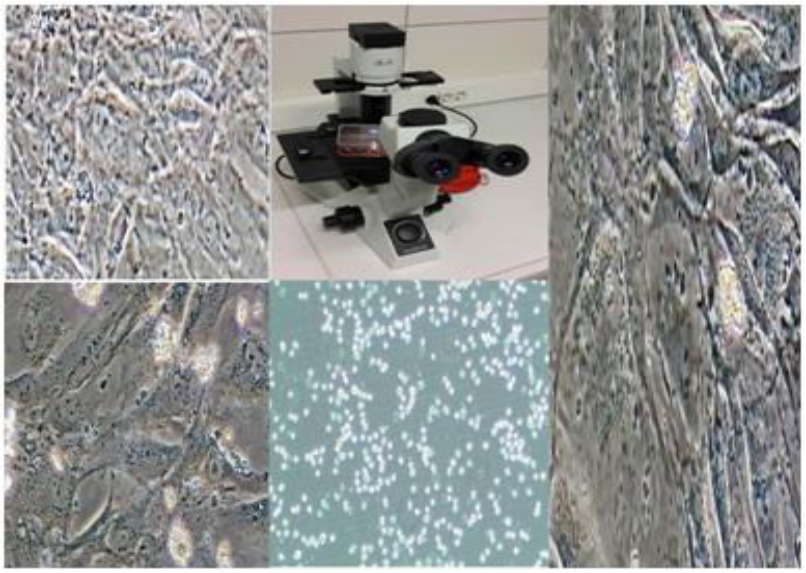

Figure 3. Due to the inability of the trypan blue dye to penetrate into the membrane of viable cells, healthy and viable cells have the small and refractile images and also have more rounded morphology compared to dead cells. In dead cells, due to the penetration of the trypan blue dye into the cell membrane, the cell sizes are larger than the living cells and are generally coloured in dark blue tones in general.

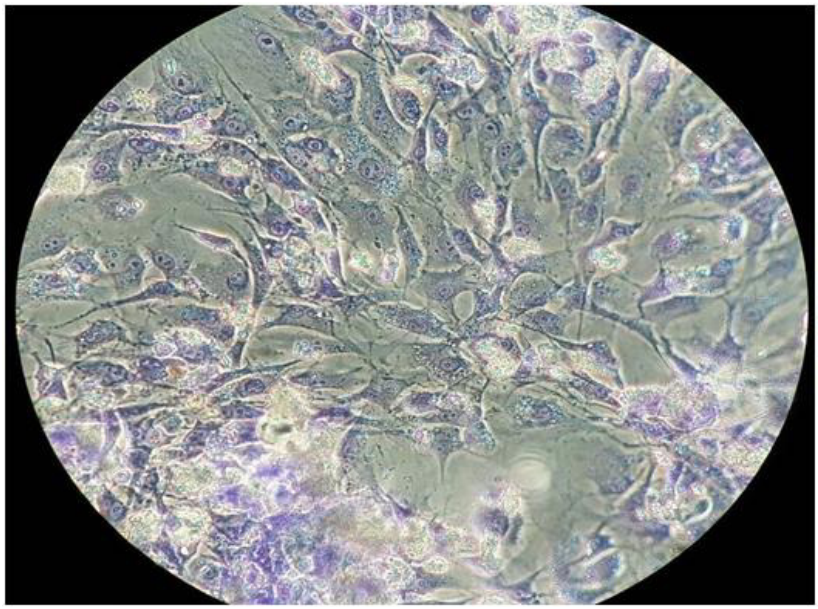

Figure 4. The images of the healthy and proliferating cell stuck to the floor and the formations related to the extracellular matrix extensions.

Inverted light microscopy analysis

Before starting the process, ocular and lenses of all microscopes should be cleaned with 70% ethanol. Cell organisms are micro-photographed at various magnifications (4X, 10X, 20X, and 40X) under inverted light microscopy before they are taken to containers where analysis will take place. To do this, the flasks should be placed on the microscope table without shaking in order to prevent contamination. With 4X magnification, the surface area of the flask covered by cells is assessed (confluency) and microphotographs are recorded at 10, 20 and 40X magnification, then cell proliferation and viability are assessed (Figure 4).

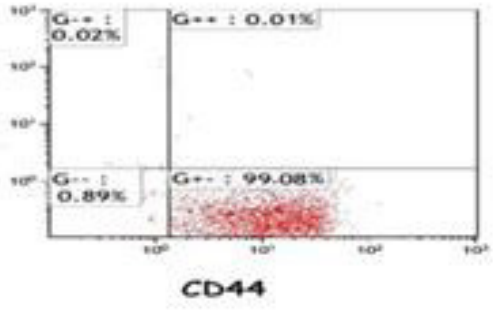

Figure 5. Quadrant image of the positive population to be displayed.

Immunoflowcytometric analyses

With the aid of immunoflowcytometry device, cell surface molecule expression levels at different time points are evaluated. Samples removed from the surface and transferred to the cytometry tubes are centrifuged at 4°C and 2000 rpm consecutively twice for 5 minutes. After centrifugation, the supernatants are discarded. The pellet located in the floor of the tube is resuspended with CCMC. Cell suspensions, in which appropriate isotype controls are added, and cluster of differentiation (CD) monoclonal antibodies bound to fluoresce in isothiocyanate (FITC) orphycoerythrin (PE) mixture are prepared. This cell suspension is incubated in the dark at 22.4°C for 25 minutes. Following incubation, centrifugation is carried out at 4°C and 1300 rpm consecutively twice for 5 minutes after the addition of PBS, pH 7.4 and containing 0.1% sodium azide. Then cells are resuspended and evaluated by the device's software program (Figure 5).

MTT cell viability, toxicity and proliferation assays

Cell viability and proliferation values can be evaluated in an ultraviolet spectrophotometer or in an ELISA-plate reader. Microplate measurement is based on measurements of the metabolic activity by comparing absorbance values from the negative controls to the values of the test group. The absorbance value from the control group is considered as 100% cell viability. During these assays, mitochondrial activities of cells can be assessed with commercial kits using a colorimetric substrate such as MTT. The tetrazolium salt contained in these kits is used for the quantitation of cell proliferation and cytotoxicity. This salt is broken down by dehydrogenase enzymes and transformed into insoluble formazan crystals that resemble a color between thiazole blue and purple based on mitochondrial activity of the living cells. There are no formazan crystals in the presence of dead cells.

In the dark environment CCMC, is removed from each well, including a control well with the aid of a pipette or gun or Pasteur pipette at the desired time intervals in the experimental setup and 500 µL of the culture medium solution containing MTT(3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide)and DMEM (1:6) should be added to each well. To halt the colour reaction after approximately three hours of incubation, 25 µL of 10% SDS solution should be added to each well (Dogan et al., 2016; Gokce et al., 2012; Gumustas et al., 2016; Gumustas et al., 2017; Guzelant et al., 2017; Isyar et al., 2015; Isyar et al., 2016; Karaarslan et al., 2018; Komur et al., 2017; Oznam et al., 2017; Topuk et al., 2017).

At least 200 µL of the MTT solutions contained in the wells are then transferred to 96-well microplates and samples are read at 490 nanometers (OD). The viable cell proliferation in cultures is calculated as % by the following formula; Proliferation = Test (Experiment Group OD) / (Control OD) X100.In this way, mitochondrial activity of the living cells in the culture medium can be easily quantified.

Preparation of AO / PI stain applied to cell cultures

Alternatively, the ratio of dead cells to viable cells can be evaluated with fluorescence images obtained after AO/PI staining. To measure cell viability, nucleic acid binding sites can also be stained with Acridine orange (AO)/Propidium iodide (PI).AO stains all nucleated cells, both live and dead, and produces a green fluorescence. PI, on the other hand, penetrates the dead cells with poor membrane integrity, stains nucleated cells and produces a red fluorescence (Akyuva et al., 2017; Karaarslan et al., 2018). AO/PI stain can be prepared using 10g Na-EDTA, 4 mg PI, 50 mL10% FBS, 4mg AO (dissolved in 2 mL99% Ethanol). They are mixed well. Then, sterile distillated water is added to reach 200 mL as the final

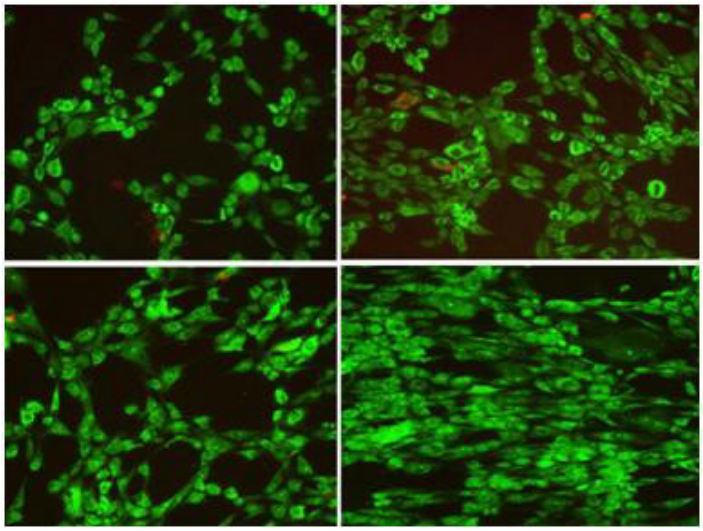

Figure 6. AO stains all nucleotide cells and produces green fluorescence. PI was able to penetrate the dead cells with poor membrane integrity and stained nucleotide cells and produced red fluorescence. As a result, nucleotide cells produced green fluorescence, and dead cells produced red fluorescence.

volume (Akyuva et al., 2017; Karaarslan et al., 2018), (Figure 6).

Environmental scanning electron microscope

The visualization of the cells isolated from human tissues *in vitro* and matrix structures, such as extracellular matrix can be performed by devices such as scanning electron microscopy (SEM) or ESEM. These two devices are different from each other. The samples to be displayed on ESEM do not need to be coated with gold or silver paints like SEM (Isyar et al., 2015; Oznam et al., 2017; Guzelant et al., 2017).

After CCMC in the containers, involving the cells, is discarded, the fixation of the samples should be ensured. To do this, a solution containing 97.5-ml of cacodylate buffer and 2.5-ml of glutaraldehyde is added to the cell-containing wells to cover the samples. The glutaraldehyde fixation solution is removed from the containers with the help of a pipette gun /Pasteur pipette over the samples, which have been kept at room temperature for 2 hours. The samples are then washed at least three times with pure cacodylate buffer and transferred to the device for imaging. Samples can be stored in the fridge at 4°C by adding cacodylate buffer until the measurement time.

qRT-PCR analyses

The qRT-PCR method is based on the enzymatic synthesis of two primers-restricted genes using a pair of synthetic oligonucleotide primers complementary to the base sequences in this region, and specific for the two ends of the region to be amplified. In the process steps, denaturation occurs at 94°C-98°C, annealing occurs at 37 ° C-65 ° C and extension occurs at 72°C.qRT-PCR is performed in two steps. The first step is the complementary DNA synthesis from RNA, which is known as reverse transcription. In the second step, complementary DNA is amplified by standard PCR (Karaarslan et al., 2018). Analyses are carried out using commercial kits specific to markers to be measured.

RESULTS

As a result of database screening, 419 studies were found to be potentially relevant. Of these, 326 studies were associated with NPCs and that 93 studies were associated with AFCs. 12 non-clinical reviews related to the NPCs proliferation and 2 reviews related to the AFCs proliferation were excluded from the study. When the remaining full texts of the 404 articles were examined, no controlled, blind, randomized, and/or comparative research was found that met the inclusion criteria. Therefore, the results could not be presented as descriptive statistics, in terms of frequency and/or mean ± standard deviation.

In the field of regenerative medicine, especially in the repair of the degenerated disc tissue, cellular treatments have been the centre of interest. However, to be used in cellular therapy, cells must survive upon injection /transplantation and differentiate into the target cell. Therefore, it is important to know how healthy cells can

be propagated *in vitro* (Gökçe et al., 2014; Isyar et al., 2016; Karaarslan et al., 2018).

Today, researchers have begun to use cellular-based experimental methods thanks to the rapid advances in tissue engineering, pharmaceutical technology and pharmaco-molecular fields. These methods are used for the diagnosis of many hereditary diseases, as well as in research on biocompatibility of materials, used in the field of neurosurgery. In addition, similar techniques are used to assess cellular toxicity of any marketed drug (Akyuva et al., 2017; Oznam et al., 2017; Sun et al., 2016).

Cell culture also plays a significant role in the repair of damaged tissues, in silencing of the damaged genome with oligonucleotides, such as shRNA/siRNA/miRNA, or in research performed to overexpress missing genes (Chen et al., 2017; Topuk et al., 2017).

Although cell culture applications are limited to major surgical fields, it is likely that such studies will take place in highly specialized fields, including neurosurgery, in the next few years. For this reason, it is necessary that all researchers, especially surgeons, working in the field of neurosurgery, should be aware of these new cellular culture studies and experimental setups.

DISCUSSION

It is emphasized in literature that components of the media for mammalian cell culture may contain amino acids, vitamins, salts, glucose, antibiotics and serum. The needs of the cells in culture should be determined depending on the purpose of the research. Media may vary based on cells type, their adaptability and organism, to ensure cell viability and cell proliferation. More importantly, studies have also reported the necessity of using chemical and/or biological agents to trigger the construction of the intracellular network in the proliferation and differentiation of cells (Gökçe et al., 2014; Isyar et al., 2016; Karaarslan et al., 2018). Molecular based experimental investigations using these agents bring an excessive burden on health economies. For this reason, in addition to being able to find the best method, it was also aimed to reduce this economic burden. To achieve this goal, it is necessary to pick up more effective and cheaper methods (Gökçe et al., 2014; Isyar et al., 2016; Karaarslan et al., 2018).

All xenocultures or pharmaceuticals used should be tested for infectious agents, in accordance with the American Food and Drug Administration (FDA) guidelines. In addition, bio-chemicals used in cell culture medium should be tested for endotoxin.

In some studies, in stead of mouse pharmaceutical embryonic fibroblasts, human-derived cell layers can be used to prevent cross-species xenotypic contamination or pathogenesis. However, even after such a culture, it is very difficult to distinguish between cells in the feeder layer and cells in the experimental group. It should be noted that the consumables to be used for primary cell cultures should carry the phrases *"endotoxin and cell culture tested".* Alternatively, researchers, performing primary cell culture, could use simple hydrogel systems that do not contain feeders' layers and allow cells to adhere to the surfaces of culture vessels using substrates, such as fibronectin or laminin.

From the past to the present, different methods have been used to isolate cells from tissues. In order to obtain cell populations from solid tissue, the tissue needs to be mechanically and enzymatically digested (Chen et al., 2012; Yilmaz et al., 2013). For single cell preparations, cells must additionally be separated from the surrounding extracellular matrix (Wu et al., 2011). General proteases, such as Trypsin, or tissue-specific enzymes, such as collagenases, used. The resulting cell suspension is heterogeneous in nature (Ding et al., 2012; Gökçe et al., 2014; Salehinejadet al., 2012; Wu et al., 2011).

In this study, when NPCs/AFCs cultures were prepared, type I and type II collagenase enzymes were used for proper isolation of cells from tissues that were surgically resected. MEM, which contains a minimal basic amino acid combination that must be found in cell cultures, was first described by Eagle in 1955. This formulation has been used with several modifications. RPMI-1640 has increased glucose for feeding the cells, has a suitable osmolarity and pH to sustain their viability, and contains the necessary amino acids and vitamins for their functioning. However, it is not sufficient by itself for the propagation of NP / AF or other cells (Isyar et al., 2016; Karaarslan et al., 2018). Therefore, in primary cell cultures using MEM, DMEM or RPMI-1640, extra nutritive solutions, such as FBS and ITS must be added to culture media to obtain healthy and proliferating cells (Isyar et al., 2016; Karaarslan et al., 2018).

FBS, which contains hormones, enzymes and growth factors that are necessary for growth and multiplication of the cells, is also a source of extracellular matrix proteins. Therefore, addition of FBS to the medium also ensures that the cells are held on adhesive surfaces (Isyar et al., 2016; Karaarslan et al., 2018).

When used in the CCMC, 1% PS should be used. However, during the transfer of disc tissues from the operating room to the laboratory or the washing of the disc tissues, use of 5% PS is recommended (Isyar et al., 2016; Karaarslan et al., 2018).

When literature was examined, studies carried out in this field, evaluated cell lines and tissues from animal sources (31, 32) (Provide names of references). However, there are substantial differences between human and animal tissues and the results obtained may be misleading (Akyuva et al., 2017; Gokce et al., 2012; Gumustas et al., 2017; Isyar et al., 2016; Karaarslan et al., 2018).

This study was limited to *in vitro* experiments, and we have not used the commercial cell lines and products, we exhibited in this research. In addition, the tissues we used in ourculture facilities were not of animal origin. We prepared human primary NPCs/AFCs cultures from tissues obtained by surgical resection. Therefore, we believe that our results using human cell sources may contribute to the literature.

Last year only, there were many different molecular studies with high-evidence value, carried out for intervertebral disc cell cultures (Bridgen et al., 2017; Gruber et al., 2017; Haschtmann et al., 2012; Liu et al., 2017; Quyang et al., 2017; Wang et al[a]., 2017; Wang et al[b]., 2017; Wiet et al., 2017; Xiong et al., 2017;). Wiet et al. (2017) evaluated the effects of mast cell media on NPCs, and AFCs. They analysed cell viability through Calcein/Ethidium and MTT-commercial kits. In addition, they evaluated the Interleukin-6, a pro-inflammatory and pro-angiogenic factor, ADAMTS5, a member of the disintegrin family, and protein-1 expression levels through qRT-PCR in the culture samples. They found that the medium used significantly increased the expression of the specific molecules in NPCs, but not in AFCs (Wiet et al., 2017).In another study (Wang et al., [a]2017), the role of Thymic stromal lymphopoietin (TSLP) in the pathogenesis of lumbar disc degeneration was investigated. In this study, degenerated NP tissues obtained from 77 patients and non-degenerated tissues of 21 patients suffering from lumbar fracture were examined. These samples, which were cultured with Hams F12+10% FBS, were transfected with different siRNA vectors. They used western blotting to detect TSLP expression in tissues, TSLP and type II collagen (COL2AL) in cell culture media were detected using ELISA, and apoptosis was determined using flow cytometry. Among the groups exposed to different transfection conditions of Hams F12 +10% FBS, they reported that TSLP expression was significantly higher in the degenerated NPCs cultures than the non-degenerated NPCs cultures. However, statistical tests regarding homogeneity or heterogeneity evaluations were not made.

CONCLUSIONS

For the time being, although cell culture applications are limited in the surgical field, it is likely that in the next few years, such investigations will be important in many branch studies, including neurosurgery. Therefore, it is inevitable that all researchers, especially surgeons, working in the field of neurosurgery, should be contribute to these new cellular culture studies to be performed in the experimental setups.

Conflict of Interest

None.

ACKNOWLEDGEMENTS

None.

REFERENCES

Akyuva Y,Karaarslan N, Yilmaz I, Ozbek H, Sirin DY, Gurbuz MS, Kaya YE, Kaplan N, Ates O (2017). How Scaffolds, which are Polymeric Drug Delivery Systems Allowing Controlled Release, can be Tested in Human Primary NucleusPulposus and AnnulusFibrosus Cell Cultures? MeritRes J MedMedSci. 5: 1-11.

[b] Wang Y, Yi XD, Li CD (2017). Role of thymicstromallymhopoietin in thepathogenesis of lumbardiscdegeneration. Medicine (Baltimore). 96: e7516.

Balla MM, Vemuganti GK, Kannabiran C, Honavar SG, Murthy R (2009).Phenotypiccharac- terization of retinoblastomaforthe presence of putativecancerstem-likecellmarkersbyflowcytometry. InvestOphthalmolVisSci. 50: 1506-1514.

Bridgen DT, Fearing BV, Jing L, Sanchez-Adams J, Cohan MC, Guilak F, Chen J, Setton LA (2017). Regulation of human nucleus pulposus cellsbypeptide-coupledsubstrates. ActaBiomater. 55: 100-108.

Chen FM, Zhao YM, Jin Y,Shi S (2012). Prospects for translational regenerative medicine. BiotechnolAdv. 30:658-672.

Chen J, Jia YS, Liu GZ, Sun Q, Zhang F, Ma S, Wang YJ(2017). Role of LncRNA TUG1 in intervertebral disc degeneration and nucleus pulposus cells via regulating Wnt/β-cateninsignalingpathway. BiochemBiophysResCommun. 491: 668-674.

Ding J, Xu H, Faiola F, Ma'ayan A, Wang J (2012). Oct4 links multiple epigenetic pathways to the pluripotency network. Cell Res. 22: 155-167.

Dogan M, Isyar M, Yilmaz I, Bilir B, Sirin DY, Cakmak S, Mahirogullari M (2016). Are the leading drugs against Staphylococcus aureus really toxic to cartilage? J Infect PublicHealth.9:251-258.

Gobbel GT, Choi SJ, Beier S, Niranjan A (2003). Long-term cultivation of multipotential neural stem cellsfromadultratsubependyma. Brain Res. 980:221-232.

Gokce A, Yilmaz I, Bircan R, Tonbul M, Gokay NS, Gokce C (2012).SynergisticEffect of TGF-β1 And BMP-7 on Chondrogenesis and Extracellular Matrix Synthesis: An InVitroStudy. Open Orthop J. 6: 406-413.

Gökçe A, Yılmaz I, Gökay NS, Can L, Gökçe C (2014). Doesinsulin, transferrin and selenous acid preparation effect chondrocyteproliferation? ActaOrthopTraumatol Turc.48:313-319.

Gruber HE, Marrero E, Ingram JA, Hoelscher Hoelscher GL, Hanley EN Jr (2017). Thechemokine, CXCL16, anditsreceptor, CXCR6, are constitutively expressed in human annulusfibrosus and expression of CXCL16 is up-regulated by exposure to IL-1ß in vitro. Biotech Histochem. 92: 7-14.

Gumustas F, Yilmaz I, Sirin DY, Gumustas SA, Batmaz AG, Isyar M, Akkaya S, Mahirogullari M (2017).Chondrocyteproliferation, viability and differentiation is declined following administration of methylphenidateutilized for the treatment of attention-deficit /hyperactivitydisorder. Hum ExpToxicol. 36:981-992.

Gumustas SA, Yilmaz İ, Isyar M, Sirin DY, Batmaz AG, Ugras AA, Oznam K, Ciftci Z, Mahirogullari M (2016). Assessing the negative impact of phenylalkanoic acid derivative, a frequently prescribed drug for the suppression of pain and inflammation, on the differentiation and proliferation of chondrocytes. J OrthopSurgRes. 11:70.

Guzelant AY, Isyar M, Yilmaz İ, Sirin DY, Cakmak S, Mahirogullari M (2017). Arechondrocytes damaged when rheumatologic inflam-

mation is suppressed? Drug Chem Toxicol. 40:13-23.
Haschtmann D, Ferguson SJ, Stoyanov JV (2012). BMP-2 and TGF-β3 do not prevent spontaneous degeneration in rabbitdiscexplants but induceossification of the annulusfibrosus. EurSpine J. 21: 1724-1733.
Isyar M, Gumustas SA, Yilmaz I, Sirin DY, Tosun HB, Mahirogullari M (2016).AreWeEconomicallyEfficientEnoughtoIncreasethePotential of *in Vitro* Proliferation of OsteoblastsbyMeans of Pharmaco chemicalAgents? Open Orthop J. 10: 420-430.
Isyar M, Yilmaz I, Nusran G, Guler O, Yalcin S, Mahirogullari M (2015). Safety of bioabsorbableimplants in vitro. BMC Surg. 12; 15:127.
Karaarslan N, Yilmaz I, Ozbek H, Sirin Yasar D, Kaplan N, Akyuva Y, Gonultas A, Ates O (2018). Arespecific gene expressions of extracellular matrix and nucleus pulposus affected by primary cell cultures prepared from intactor degenerative intervertebral disect issues? TurkNeurosurg. doi: 10.5137/1019-5149.JTN.22210-17.2
Komur B, Akyuva Y, Karaslan N, Isyar M, Gumustas SA, Yilmaz I, Akkaya S, Sirin DY, Mutlu CA, Batmaz AG, Guler O, Mahirogullari M (2017). Can a BiodegradableImplantedBilayeredDrug Delivery SystemLoadedwith BMP-2/BMP-12 Take an Effective Role in theBiologicalRepairProcess of Bone-TendonInjuries? A Preliminary Report. J Pharm (Cairo). 2017: 7457865.
Lijmer JG, Mol BW, Heisterkamp S, Bonsel GJ, Prins MH, van der Meulen JH, Bossuyt PM (1999). Empiricalevidence of design-relatedbias in studies of diagnostictests. JAMA. 282:1061-1066.
Lin H, Zhang W, Zhou T, Li W, Chen Z, Ji C, Zhang C, He F (2017).Mechanism of microRNA-21 regulating IL-6 inflammatory response and cell autophagy in intervertebral discdegeneration. ExpTherMed. 14:1441-1444.
Liu H, Yang SD, Xu Y, Ning SH, Wang T, Yang DL, Ding WY (2017). Protective role of 17β-estradiol on tumornecrosisfactor-α-induce dapoptosis in human nucleuspulposus cells. MolMedRep. 16: 1093-100.
Oznam K, Sirin DY, Yilmaz I, Kaya YE, Isyar M, Gumustas SA, Ozbek H, Akkaya S, Kayhan A, Mahirogullari M (2017). Iopromide- and gadopenteticacid-derived preparates used in MR arthrographymay be harmfultochondrocytes. J OrthopSurgRes. 12:98.
Quyang A, Cerchiari AE, Tang X, Liebenberg E, Alliston T, Gartner ZJ, Lotz JC (2017). Effects of cell type and configuration on anabolic and catabolic activity in 3D co-culture of mesenchymal stem cells and nucleuspulposus cells. J OrthopRes. 35: 61-73.
Salehinejad P, Alitheen NB, Ali AM, Omar AR, Mohit M, Janzamin E, Samani FS, Torshizi Z, Nematollahi-Mahani SN (2012). Comparison of dif- ferent methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton'sjelly. InVitro Cell Dev Biol Anim. 48: 75-83.
Sun Z, Luo B, Liu Z, Huang L, Liu B, Ma T, Gao B, Liu ZH, Chen YF, Huang JH, Luo Z (2016). Effect of perfluorotributylamine-enrichedalginate on nucleuspulposus cell: Implications for intervertebral discregeneration. Biomaterials. 82: 34-47.
Topuk S, Akyuva Y, Karaaslan N, Mutlu CA, Yilmaz I, Isyar M, Sirin DY, Akkaya S, Özbek H, Mahirogullari M (2017). Is it Possible to Treat Osteosarcoma Using Oligonucleotides Confined into Controlled Release Drug Delivery Systems? CurrPharmBiotechnol. 18:516-522.
Vacanti J (2010). Tissue engineering and regenerativemedicine: from first principles to state of the art. J Pediatr Surg. 45:291-294.
[a] Wang SJ, Sun ZY, Liu C, Yan P, Liang H, Huang K, Wang DG, Li Y, Tian JW (2017). High mechanical stretch stress promotes degeneration of the human nucleus pulposus cellsthrough NF-κbsignalingpathway. ZhonghuaYiXueZaZhi. 97: 1964-1969.
Wiet MG, Piscioneri A, Khan SN, Ballinger MN, Hoyland JA, Purmessur D (2017). Mast Cell-Intervertebral disc cell interactions regulate inflammation, catabolismandangiogenesis in DiscogenicBackPain. SciRep. 7: 12492.
Wu W, He Q, Li X, Zhang X, Lu A, Ge R, Zhen H, Chang AE, Li Q, Shen L (2011). Long-term cultured human neuralstem cells undergo spontaneous transformation to tumor-initiating cells. Int J BiolSci. 7: 892-901.
Xiong X, Dai L, Liang W, Zhang J, Qin S, Cao W, Ye D, Liang P, Liu Z (2017). Protectiveeffect of p53 on theviability of intervertebral disc nucleus pulposus cells under lowglucose condition. Biochem. Biophys. Res. Commun. 490:1414-1419.
Yilmaz I, Gokay NS, Bircan R, Saracoglu GV, Dervisoglu S, Gokce A (2013). How different methodologies of harvesting and analysing the samples affect the test results in determining joint mediators. Arthritis. 2013; 2013: 631959.