MERIT RESEARCH JOURNALS
www.meritresearchjournals.org

Merit Research Journal of Medicine and Medical Sciences (ISSN: 2354-323X) Vol. 5(10) pp. 477-487, October, 2017
Available online http://www.meritresearchjournals.org/mms/index.htm
Copyright © 2017 Merit Research Journals

Original Research Article

How Scaffolds, which are Polymeric Drug Delivery Systems Allowing Controlled Release, can be Tested in Human Primary Nucleus Pulposus and Annulus Fibrosus Cell Cultures?

Yener Akyuva[1], Numan Karaarslan[2*], Ibrahim Yılmaz[3], Hanefi Ozbek[4], Duygu Yasar Sırın[5], Mehmet Sabri Gurbuz[6], Yasin Emre Kaya[7], Necati Kaplan[8] and Ozkan Ates[9]

[1]M.D. Gaziosmanpasa Taksim Training and Research Hospital, Department of Neurosurgery, 34433, Istanbul, Turkey

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

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

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

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

[6]Assist. Prof. M.D., Istanbul Medeniyet University School of Medicine, Department of Neurosurgery, 34000, Istanbul, Turkey

[7]Assist. Prof. M.D., Abant Izzet Baysal University School of Medicine, Department of Orthoaedic and Traumatology, 14000, Bolu, Turkey

[8]Assist. Prof. M.D., Istanbul Rumeli University, Corlu Reyap Hospital, Department of Neurosurgery, 59860, Tekirdag, Turkey

[9]Prof. M.D., EsenyurtUniversity, EsencanHospital, Department of Neurosurgery, 34517, Istanbul, Turkey

***Corresponding Author's Émail: numikara@yahoo.com**
Pbx: +9028 2250 5500

Abstract

Promising pharmacological treatment of disk degeneration depends on development of appropriate materials and obtaining viable and proliferative nucleus pulposus/annulus fibrosus tissues with preservation of extracellular matrix compounds. This study aims to explain how drug and/or similar material impregnated polymeric scaffolds can be tested using primary cell cultures derived from human nucleus pulposus and annulus fibrosus tissues at pharmacomolecular level. For this purpose; we first explained how to prepare human primary cell culture setups. We remarked the physicochemical parameters for testing drugs and scaffolds containing drug-like substances. How to conduct the molecular analyzes of samples of cell cultures was explained. In this study; we explained very basically how to make in vitro analyzes for the development of the ideal drugs and/or polymeric biomaterials that can be used in clinical practice for the purpose of preventing disc degeneration. Moreover, medical pharmacologists, orthopedic and brain surgeons taking part in such studies can set up and analyze their own studies.

Keywords: Annulus fibrosus/ nucleus pulposus culture, controlled release, polymeric scaffolds

INTRODUCTION

Scientists have long been aware that a complete repair of the damaged tissue is not possible. Although tissue repair is accepted possible to some extent, it is not possible for a damaged tissue to regain its original form (Hunter's et al., 1995).

It is not possible to obtain the original tissues following a pathologic process, since some functional losses occur afterwards and physiological permanency is disturbed. Consequently, patients, their relatives and economies of countries are affected in terms of physical, emotional and workforce problems (Uddin's et al., 2015).

Intervertebral disc degeneration (IVD) is one of the most commonly encountered degenerative processes in skeleton system. Nutritional inadequacies that are dependent on oxygen and glucose deficiency and additional genetic factors are among the factors facilitating IVD. Moreover, damages are seen as a result of decrease in the diffusion of effluents and increase in disc volume which are caused by mechanic overload, vibration and trauma. In addition to these, smoking and/or decrease in blood stream resulting from vascular diseases or idiopathic endplate calcification are among the burning issues. Lactate increase, pH decrease, proteoglycane fragmentation and loss, protein modification, and phenotype change may occur after calcification, which eventually leads to matrix destruction. Afterwards, scratch and rupture formation, granular changes and mucoid degeneration may occur (Postacchini's et al., 1984; Errington's et al., 1998; Bruehlmann's et al., 2002). Apart from pathologic process, there may be ordinary changes as well as different degenerative or regenerative changes. Among these changes; IVD plays the major role in the development of osteoarthritis of facet joints and degeneration of ligamentumflavum (Christoph's et al., 2011; Yoshiiwa's et al., 2016; Lipson's et al., 1981).

Since IVD, a result of Annulus fibrosus (AF) and/or nucleus pulposus (NP) damages, does not respond to conservative treatments the process usually ends up with spinal surgeries. These surgeries are performed for the purpose of relieving the pain resulting from nerve root compression. Besides, they are performed for the recovery of neurologic deficits and usually including fusion and/or decompression in instable segments. Today, root decompression is performed by microdiscectomy for the patients with simple degenerated lumbar disc hernia. Decompressive laminectomy is performed in patients with central canal stenosis. Transpedicular and/or interbody fusion is a treatment modality preferred in the treatment of instable axial pain. However, the current surgical methods are still questionable when their long term results are concerned (Best's et al., 2015).

For this very reason cell-free scaffold, 3D biocomposite scaffold and scaffolds with stem cells and/or growth factors have been in the center of interest. Drug delivery systems are still being tested in the researches and studies concerning the systems with controlled drug and/or gene release (Gumustas's et al., 2016; Dogan's et al., 2016; Isyar's et al., 2015; Isyar's et al., 2016; Yılmaz's et al., 2016; Isyar's et al., 2016; Gumustas's et al., 2016; Guzelant's et al., 2017; Isyar's et al., 2015; Gumustas's et al., 2016).

It is known that scaffolds support cell and tissue formation, allow efficient diffusion of biochemical molecules, dissolve in a certain time interval and leave its place to the host tissue, by providing nuclear assembly, cell adhesion and diffusion of nutrients into cells.

In this compilation, we aimed to show how polymeric scaffolds, which can be locally targeted by the controlled release of drug and/ or growth factors, are tested at the pharmaco-molecular level.

METHODS

Approval of Local Ethics Board is required before the setup of primary cell cultures from tissues. Afterwards, written informed consent forms are obtained from the patients. All criteria in declaration of Helsinki are measured up at the start and end of experiments at all steps.

A clear explanation of the tissues to be used in the study, particularly from which cases they are obtained is essential. The cases with malnutrition, organ failure such as kidney and/or liver, HIV, bone marrow hypoplasia, leucopenia, thrombocytopenia, anemia were excluded from the study. In addition, it is important to exclude the cases who are allergic to drug or egg protein and as well as pregnant and nursing women.

Tissues to be used in the preparation of primary cell cultures for nucleus pulposus cells (NPCs) can be obtained from the operated patients presenting with lumbar and/or leg pain complaint(s) (Sive's et al., 2002). Following hematologic, biochemical, physical and neurologic examinations, patients with lumbar disc hernia can be chosen. However, magnetic resonance images (MRI) and lumbar roentgenograms of the cases are routinely needed. In these cases, it is important to know whether there are degenerated disc hernias with sequestrated fragment compressing nerve root and spinal cord (Adams's et al., 2006).

Considering Pfirrmann Classification, rating in disc degeneration is made through T2-weighted magnetic resonance imaging (Hanley's et al., 1996). The cases should also be controlled whether they have sequestrated disc hernia, intra/subligamentous extrusion, according to the modification classifications adopted by The International Society for the Study of the Lumbar Spine and The International Society for the Study of the

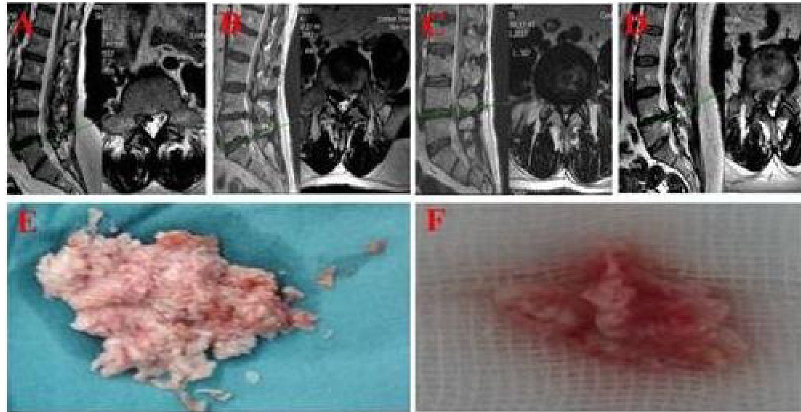

Figure 1.A, B, C, and D. T-2 weighted sagittal and axial lumbar MRI scans of the patients undergoing lumbar microdiscectomy revealing herniated intervertebral disc degenerations of subligamentous, intraligamentous, supraligamentous and sequestrated types respectyively. ***E;*** The tissue sample obtained solely from NP. **F;** The tissue sample containing both NP and AF.

Lumbar Spine Surgical Indication and Techniques *(Figure 1A, B, C, and D).*

After approval of Local Ethics Board and written informed consent forms are obtained from the cases undergoing operation(s), and standard lumbar micro discectomy procedure is performed.

Patients are positioned in neutral and prone position before the endotracheal intubation and the antisepsis of the surgical area is provided by povidone iodine. The area is covered to provide a sterile environment. A 2 cm midline skin incision is enough to reach the herniated disc segment. Unilateral hemi partial laminectomy and partialfacetectomy are performed under the microscope.

Following extruded or sequestrated nucleus pulposus tissue is taken, disc is penetrated by means of disc punch. Annulus fibrosus (AF) is excised in 1cm depth, whereas nucleus pulposus is excised in 2-3 cm depth. During lumbar micro discectomy, the resected tissues are taken into sterile falcon tubes in order to be used for NPC/AFC cultures *(Figure 1E, F).*

After these, tissues are taken into falcon tubes with sterile caps and containing freshly prepared transfer medium. The transfer should be made at 4°C in suitable conditions and completed between two and six hours.

It is important to use transwell chamber depth during the transfer of controlled drug delivery system into culture tubes. Because, it enables drug delivery system which has not completed its task to leave the environment.

Physicochemical test of polymeric drug delivery systems which allow controlled release

Swelling test, pH, heat, and UV spectrophotometric analyses

We aimed to calculate molecular weight of inflating polymer between cross connections in our delivery system. Also we aimed to make the calculation based on a theory such as Flory Rehenner. In practice, this is done by swelling test. Hydrogel samples should be fractionated into pieces with a minimum of 4.5mm inner diameter, a minimum of 1.5 mm thickness and a minimum of 10 mm length in order to calculate inflation balance in ultra-pure water. The samples are frozen for 6 hours at -20° C and then waited for 6 hours at 37±0.5 °C (Ciftci's et al., 2015).

Dry weight of the samples is reported after the samples are weighed. Afterwards, the polymeric samples should be put into beakers with 25 ml pure water and waited for 4 hours with at least 20 min intervals. The samples should be separated from their environment by means of filtration before they are put into beakers. The samples are taken out in every 20 minutes and dried using a blotting paper. Then the weight of the dried samples are weighed and reported.

The diameters of the hydrogel samples are measured using a digital caliper. Hydration percentage of the polymeric drug delivery system is calculated as follows: % Dynamic weight change (hydration percentage) = (W2-W1/W1) ×100 (Ciftci's et al., 2015). W2 = symbolizes the weight of hydrogen fragments in water and W1 = symbolizes first dry weight.

While determining pharmacokinetics parameters pertaining to controlled release, release experiments can be done in phosphate tampon (PBS; pH=7,4). The absorbance changes during the release of the imbued growth factors and/or drugs were observed at 37.4 °C using UV-Spectrophotometer. Hence PBS solution containing 0.1N 0.9% isotonic sodium chloride solution is to be prepared. The design is added to this mixture. The design material which contains drug and/or growth factors are evaluated as separate layers. They are waited in beakers for at least eight days, yet PBS solution is changed twice a day q12h.

While PBS is changed in the beakers, viscose liquid samples are taken into one millimeter Eppendorf caps. They are spectrophotometrically scanned. Main factor and auxiliary products are measured which are used in PBS and drug delivery system. However, it is important that spectrophotometric device be blank to PBS solution content and drug delivery system. Thereby the device which is blank to the main structure of the design and PBS content can be used for measurements of the release of drug and/or growth factors.

Time graphs should be prepared considering absorbance. The released drug/growth factor amount is subtracted from the drug/growth factor loaded into hydrogel. By this means it is absorbed as it stays in polymeric scaffold drug delivery system. Drug/growth factor amounts can also be calculated (Ciftci's et al., 2015).

After the swelling equilibrium, the diameters of the polymeric scaffolds were recorded. Afterwards, the diameters were measured and the averages of the obtained values were calculated in 25, 60 and 80°C, respectively. By using different concentrations of HCl and NaOH, pH adjusted ultra-pure water was prepared and the changes in the diameter of these scaffold samples were recorded.

Environmental Scanning Electron Microscope (ESEM) and energy dispersive x ray spectrometry analyses

After media are taken out, a mixture comprised of 97.5 ml cacodylate tampon buffer and 2.5 ml glutaraldehyde is prepared. 2.5 % glutaraldehyde solution should be added to samples to cover them.

After the samples are waited for 2 hours at room temperature and the mixture with glutaraldehyde is taken out by a pipette, samples are to be washed for 3 times by means of cacodylate buffer. The frozen samples at -20℃ are waited in a freeze-dryer for a night. After they are dried, they can be examined.

The samples which are prepared for ESEM for extra should be applied to some processes for the fixation of cells for which 8 % glutaraldehyde and cacodylate buffer solution is needed. It is taken from the glutaraldehyde solution in the sterile laminar cabin and is diluted from 8 to 2.5 % by sterile distillated water. The amount taken from glutaraldehyde solution is 31.25 ml and put into graduated cylinder. Afterwards, approximately 100 ml of distillated water is added. The solution is added to sterile glass bottles and shaken until they are homogenized. 97.5 ml is taken from cacodylate buffer solution. Next, 2.5 ml of2.5 % glutaraldehyde solution is added and shaken.

This previously prepared solution is added to all cell-containing plates so as to cover the samples. Cells are waited for 120 minutes at room temperature. Cacodylate buffer and glutaraldehyde mixture is taken away from the environment by means of a pipettor. The samples with pure cacodylate buffer which does not contain glutaraldehyde are washed for 3 times. Following the last wash, cacodylate buffer is added into the samples to cover their plates. Then, they are packed and waited at 4°C until they are transferred to SEM. Also, the samples can be packed with aluminum foil till SEM analysis (Guzelant's et al., 2017; Isyar's et al., 2015; Ciftci's et al., 2015).

EDAX analyses of implant samples that do not contain design could be made, which enables to observe whether the drug delivery system provides an appropriate environment (Isyar's et al., 2015).

The number of carbon atoms in the designed material will be determined. By this means, whether they are determined as chemicals will be identified?. In the same breath, it will be observed whether it serves to be an appropriate growth environment for cells.

Test of Polymeric Drug Delivery Systems, which allow controlled release, in human primary NPCs and AFCs cultures on Pharma-molecular level

The prepared NPCs/AFCs cultures should be placed into plates that are suitable for analyses. The reproduced primary cells in flasks should be passaged, in the presence of Tripan Blue, by means of a hemocytometer such as Thoma lam.

An analysis could be performed based on the principle of putting lamellar on lam with counting chambers. In the lam, there are 1 mm scraped squares and a surface comprised of smaller squares. They are taken from culture plates by means of trypsinization of cells. The suspended cells are homogenized for 2 or 3 times. The surface of the plates should be covered namely to be confluent in order for the passage process. 2ml trypsin for 100 mm petri, 1 ml trypsin for 35 mm petri and 0,5 ml trypsin for 35 mm petri are used. While calculating cell numbers, 10 µl is taken from cell suspension from 1 ml diluted culture medium. It is put into Eppendorf tube then 90 µl Trypan blue stain is added and mixed (Isyar's et al., 2016; Ciftci's et al., 2015).

This mixture is put into Thoma lam and calculations are made in order to find how many millions there are in 1ml medium. After the determination of planting number, the cells are put into flask, well plate and petri.

In the calculation *A x SF x16 x 10.000* formula is used. The symbolizations are as follow: *A:* Cell number in a middle sized square, *SF:* Dilution factor, *16:* multiplier to reach square result as there are 16 middle sized squares *10.000:* invariable used to convert the count result of 0,1 mm^{3}' into a number in 1mL' to obtain a standard result (Isyar's et al., 2016; Ciftci's et al., 2015).

The important point is that as blue stain cannot penetrate into the membrane of living cells, the views of cells are small, retractile and in roundish shape

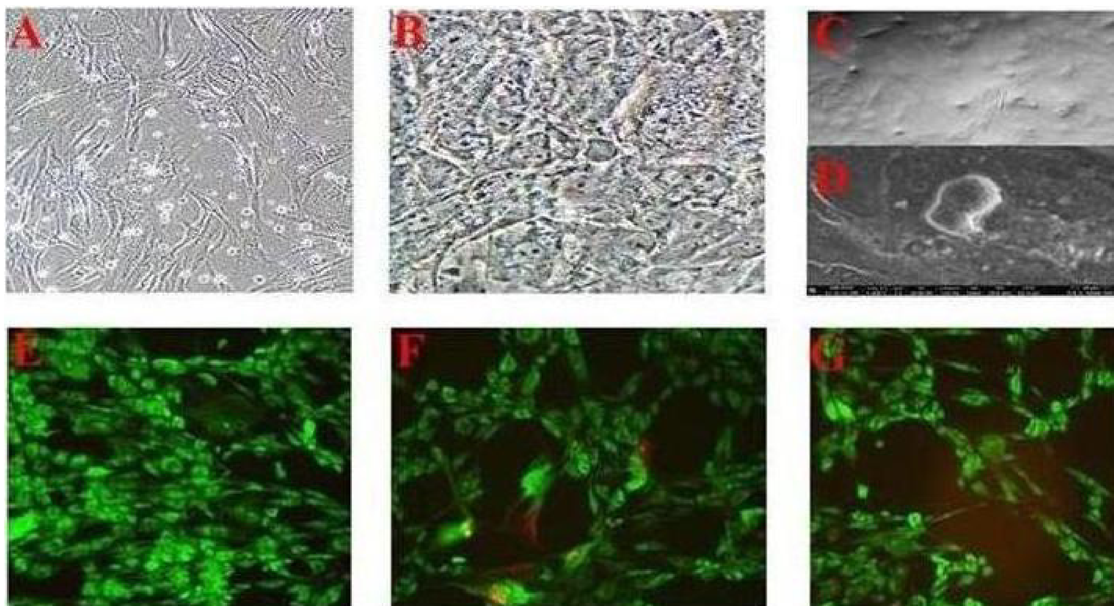

Figure 2.A, B. Inverted light microscopy (x10, x40 magnification), and **C, D.** Environmental scanning electron microscopy (x500, x5000 magnification), **E, F, and G;** Acridine orange/propidium iodide stain images.

morphology when compared to dead cells which are bigger and mostly in thiazole color (Gumustas's et al., 2016; Dogan's et al., 2016; Isyar's et al., 2015; Isyar's et al., 2016; Isyar's et al., 2016; Gumustas's et al., 2016; Guzelant's et al., 2017; Isyar's et al., 2015; Ciftci's et al., 2015). Cells that are counted are to be transferred to the suitable plates.

Inverted light Microscopy

Before the process, all ocular and lenses of microscopes should be cleaned using 70% ethanol. Flasks are gently placed into the microscope platform paying attention not to spill the culture medium on the scape of flasks.

Firstly, it should be observed how much of the surface of the flask is covered by ×4 magnification. Later on, images at ×10, ×20 and ×40 magnifications are recorded, respectively, so it can be understood how much of cells are sticked to the base and whether they are healthy or proliferous *(Figure 2A, B).*

Environmental Scanning Electron Microscopy (ESEM)

As mentioned in section in which chemical features of scaffolds are told, cell surface morphology is evaluated with the same method (Gumustas's et al., 2016; Dogan's et al., 2016; Isyar's et al., 2015; Isyar's et al., 2016; Isyar's et al., 2016; Gumustas's et al., 2016; Guzelant's et al., 2017; Isyar's et al., 2015; Ciftci's et al., 2015). Hence glutaraldehyde- cacodylate buffer solution is added to the plates to cover them. The cells should be waited for 130 minutes at room temperature.

Cacodylate buffer mixture with glutaraldehyde is taken from the environment by means of a pipettor. It is washed three times by pure cacodylate that does not contain glutaraldehyde. Following the last wash, cacodylate buffer is added into the samples to cover their plates. Then, they are packed and waited at 4°C in the fridge until they are transferred to SEM (*Figure 2C, D).*

Cell toxicity, viability, and proliferation analysis: Enzyme linked immunosorbent assay (ELISA)

Viability tests were performed via a commercial MTT kit (3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (Commercial Cell Proliferation Assay kit) using the manufacturer's instruction. The kit's working principle is explained as follows: tetrazolium ring is cleaved by mitochondrial dehydrogenase enzymes yielding blue formazan crystals, which do not occur in dead cells. MTT analyses were performed before (control group=Group I) and after the drug(s) and or scaffold were added (Group II and/or III) using an ELISA (O.D. 540 nm) spectrophotometric microplate reader. Viability of the control group was assumed to be 100% before the transfer of drugs and/or scaffold (0h) to the culture medium. 24 h after the administration of the drugs and/or scaffold, viability absorbance of the cells was recorded as nm. Then, proliferation analyses for hours (24h-48h) were recorded.

To determine cell viability and confirm MTT results nucleic acid binding dyes Acridine orange (AO)/Propidium iodide (PI) were used. AO stain all nucleated cells both live and dead and generate green

fluorescence. PI can only penetrate to dead cells with poor membrane integrity, and stain nucleated cells to generate red fluorescence. Cells stained with both AO/PI all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red.

Preparation and application of the AO/PI

AO/PI stain prepared with 10g Na-EDTA, 4 mg PI, 50 ml Fetal calf serum, 4 mg AO (dissolved in 2ml 99% ETOH) is mixed well and sterile distilled water is added to reach a 200 ml of final volume.

With the nucleic acid binding dyes AO and PI cell viability is determined accurately. AO is an intercalating dye to permeate both living and dead cells. AO stain all nucleated cells and generate green fluorescence. PI can only enter dead cells with poor membrane integrity, and stain all dead nucleated cells to generate red fluorescence. Cells are stained with both AO/ PI. Ultimately, all living nucleated cells fluoresce green and all dead nucleated cells fluoresce red *(Figure 2E, F, and G).*

RESULTS

It appears that imaging techniques of these studies are similar and the commercial kits that are used are expensive kits. What's more, while there are studies regarding drug delivery system with controlled release, some of them have complicating points in terms of the calculation of the release amount of pharmacological agents. Others include inadequate calculations. Cumulative release amounts are calculated in some of the studies whereas release amounts cannot be calculated in some other studies.

DISCUSSION

Recently IVD has been considered as one of the main reasons for lumbar pain which lead to various lumbar pathologies. Correspondingly, it puts a strain on health economies (Handley's et al., 2015). These pathologies involve an increasing degeneration that results from biochemical degradation of NP or herniation. The situation eventuates in disc dysfunction by inhibiting disc movement. For this very reason, in the treatment of degenerative intervertebral discs, tissue engineering has stepped in in order to cease degenerative process or provide regeneration (Li's et al., 2016; Chan's et al., 2013; Renani's et al., 2012; Feng's et al., 2013; Bhattacharjee's et al., 2012; Koepsell's et al., 2011; Yeganegi's et al., 2010; Alini's et al., 2002; Jalani's et al., 2016; Cheng's et al., 2011; Willems's et al., 2015; Murab's et al., 2015).

Current approaches in disc degeneration are surgical and conservative methods which aim to prevent disc movement. Therefore studies concerning NP/AF of the cases with IVD have been conducted recently, such as tissue engineering with bio scaffolds in order for the protection of microstructure, biochemical, mechanical features and cell vitality.

The aim of these researches in the long term is to provide biological regeneration of NPCs/AFCs, whose modality of treatment is mainly in inner part, so experiments regarding regeneration through the use injectable material by endoscopy have been the center of interest. NP tissue engineering is a new method of early stage disc degeneration. Cell based treatments may develop the restoration of bio mechanic function of AF tissue and its own repair capacity. In addition to this, it plays an important role in choosing appropriate cell source and design of scaffold (Li's et al., 2016; Chan's et al., 2013; Renani's et al., 2012; Feng's et al., 2013; Bhattacharjee's et al., 2012; Koepsell's et al., 2011; Yeganegi's et al., 2010; Alini's et al., 2002; Jalani's et al., 2016; Cheng's et al., 2011; Willems's et al., 2015; Murab's et al., 2015).

This review aims to explain that scaffolds, which are studied to ensure the cell aggregation, adhesion of cell, diffusion of nutrients into cells, and to be locally targeted by the controlled release of drug and/ or growth factors, support cell and tissue formation, permit the effective diffusion of biochemical molecules, dissolve after a certain time leaving its place to the home tissue, how can be tested at the pharmaco-molecular level.

Zhou et al. stated that type 2 collagen has a part in the differential of adipose-derived stem cells (ADSCs) into NP quasi phenotype. From this point forth, they designed a 3D collagen scaffold using N,N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide and N-hydroxysuccinimide (EDAC/NHS) molecules with the purpose of increasing type 2 collagen. They reported that cross-linked collagen scaffolds have higher biological stability and spatial structure than other scaffolds. While activity level of scaffolds is evaluated, physicochemical characterizations such as porosity, biodegradation, microstructure are analyzed as well as biological characters such as cytotoxicity, cell proliferation, expression of relevant genes and proteins. They compared protein and gene expressions pertaining to aggrecan SOX9 and type 2 collagen. Thanks to molecular techniques, they reported that frozen-dried collagen scaffolds which are cross linked with EDAC/NHS are the best 3D scaffolds regarding ADSC proliferation and differentiation of these cells to NP quasi phenotype (Zhou's et al., 2016).

Kumar et al. have tested a hydrogel scaffold based on injectable and photo curable synthetic polymers [(pHEMA-co-APMA grafted with polyamidoamine (PAA)]. They indicated that human mesenchymal stem cells (hMSC) are potential agents to be transformed into NP

phenotype under hypoxic conditions. By means of molecular techniques they proved that these cells can be used in order to improve NP tissue function and mechanical characters.

For this purpose; they have conducted their studies in chondrogenic culture medias under hypoxic conditions concerning the expression levels of aggrecan and type-2 collagen proteins. (Kumar’s et al., 2014).

Smith et al. indicated that IVD is an important cause of back pain and new regenerative studies are needed to restore the natural tissue structure and mechanic functions. In their research, they used a hydrogel scaffold with 3D interpenetration which also contains dextran, chitosan and teleostean. They probed therapeutic potential in NP regeneration through biomedical, cytotoxicity and tissue engineering studies. They evaluated biomedical features of the design based on gelation time function. They expressed that mechanic features of scaffold which are evaluated in limited and unlimited compression are similar to natural human NP features. In order to evaluate the disc under physiological load, they injected toluidine-blue-labeled hydrogel into spine segment which had been obtained from human cadaver by nucleotomy. As a result of the analysis, they reported that implanted material was not given off and hydrogel was well placed into natural NP. By means of commercial kits it is seen that scaffold is not toxic to NPCs and it even increases vitality and proliferation. In order to evaluate cell-mediated matrix production of scaffold, they expressed that they planted structures with mesenchymal stem cells (MSCs) and cultured them for 42 days under prochondrogenic conditions. They examined specific markers (mRNA upregulation of aggrecan and type 2 collagen) and maintained that increased GAG, collagen content and mechanic features were signs of MSCs differential as chondrogenic(Smith’s et al., 2014).

Xu et al. aimed to produce a new composite scaffold containing acellular demineralized matrix/ acellular nucleus pulposus. They detected physicochemical features of a scaffold and examined its use in intervertebral disc tissue engineering and its feasibility. They analyzed scaffold by means of Hematoxylin and Eosin staining and scanning electron microscope. Besides, they carried out porosity measures, examined absorption rates and evaluated them in terms of compressive elastic modulus. What’s more they used adipose derived stem cells (ADSCs) cultures in various concentrations of scaffold extracts for the purpose of evaluating cytotoxicity. They indicated that they determined the vitality of ADSCs through Live/Dead staining method. Thanks to molecular techniques they maintained that new composite scaffold had enough pore diameters, its bio mechanic features were similar to natural intervertebral disc, it was not cytotoxic, but biocompatible and it was appropriate for tissue engineering (Zhou’s et al., 2016).

Liang et al. reported that they aimed to study the feasibility of nanostructured 3D poly(lactide-co-glycolide) (PLGA) constructs, in which growth factor is imbued and heparin/poly(L-lysine) nanoparticles and dexamethasone (DEX) are loaded. They indicated that scaffolds were aimed to be used effectively in NP tissue engineering. With the results, they reported that microsphere constructs were able to realize basic fibroblast growth factor and DEX releases simultaneously. They claimed that dual bead microspheres do not result in toxicity. Moreover, they maintained that rat mesenchymal stem cells (rMSCs) affect proliferation positively by means of lactate dehydrogenase and CCK-8 analyses. In the research carried out with in vitro cell cultures, they observed that rMCSs-scaffold hybrids contain significantly high sulfated GAG/DNA and type 2 collagen II when compared to the control groups. Also, by means of real time PCR analysis they reported that matrix protein expressions such as type II collagen, aggrecan and versican were significantly higher in rMCSs-scaffold when compared to the control groups. They used molecular techniques in their research and indicated that Dex/bFGF PLGA microspheres might be used as scaffolds to contribute to the growth of rMSCs and differentiation to NP quasi cells. Besides, they concluded that these scaffolds might decrease inflammatory response in IVD tissue engineering(Liang’s et al., 2012).

Cheng et al. added gelatin molecules into thermosensitive chitosan/beta-glycerol phosphate (C/GP) disodium salt hydrogels to form chitosan/gelatin/beta-glycerol phosphate (C/G/GP) disodium salt hydrogels, which are applied as cell delivery for NP regeneration. They analyzed C/G/GP hydrogel endurance, gelation time and gelation warmth through a rheometer. They obtained NPSs from New white rabbits in New Zealand and cultured them in C/G/GP hydrogels on monolayers. They analyzed cell vitality, cytotoxicity based on material, cell proliferation, sulfated glycosaminoglycan production, anabolic/catabolic gene expressions and extracellular matrix-related gene expressions pertaining to NP cells. Thanks to molecular techniques, they concluded that C/G/GP hydrogels are suitable for NPCs culture and could be used in minimal invasive intervertebral disc surgery in the future (Cheng’s et al., 2010).

When literature was reviewed, it was found that there were few articles with high proof *(Table 1).*

All in all, it is known that an ideal biomaterial has not yet been developed which clinically supports regeneration of NP/AF. In addition to this, developing a promising biomimetic/bio polymeric material means developing NP/AF tissues by preserving extracellular matrix components.

Table 1. Characteristics of the included studies

	Type of material	Production technique	Physico-chemical/ Pharmacokinetic testing	Biomechanical testing	The experimental setup	Molecular test/ Spesific marker	Imaging	Histopathological evaluation
Feng's et al., 2013.	Nanohydroxyapatite/ Polymercompositescaffold	3D	-	-	*In-vitro*	Hypoxia-inducible factor alpha, Sox-9, collagentype I and II collagens, DNA content, andaggrecan	Confocal fluorescence microscope.	Hematoxylin & Eosin, Fluoresceinisothiocyanateand 4'-6-diamidino-2-phenylindole
Bhattacharjee's et al., 2012.	Oriented lamellar silk fibroinhydrogel scaffold	Winding machine,	-	Tensile strength was Evaluated using a mechanical testing device, and atomic force microscopy	*In-vitro*	Cell proliferation kit, Glycosaminoglycan, Collagentype II, DNA content,	Attenuated total reflectance Fourier transform infrared Spectroscopy, Nuclear magnetic resonance, Scanningelectron microscopy.	Safranin O, Hematoxylin and Eosin.
Koepsell's et al., 2012.	Polycaprolactone fibers scaffold	3D	-	Tensile strength was evaluated using a mechanical testing device	*In-vitro*	DNA content, Sulphated Glycosaminoglycan, Total collagen content.	Scanning electron microscopy.	Safranin-O/ Picrosiriusred.
Yeganegii's et al., 2010.	Polyurethananenofiber scaffold	Electrospinning	Differential scanning calorimetry	Tensile strength was Evaluated using a mechanical testing device	*In-vitro*	-	Scanning electron microscopy	Ethidium homodimer-1.
Jalani's et al., 2016.	Chitosanhydrogelcoatedupconvertingnanoparticles scaffold	Photocleavable crosslinker	Near-infrared laser irradiation, UV-Vis absorption spectral analysis, x-ray photoelectron spectroscopy.	-	*In-vitro*	Cell viability	Transmissionelectronmicroscopy, Nuclearmagneticresonance	Alamarblueassay
Cheng's et al., 2010.	Injectable thermosensitive chitosan-gelatin-glycerolphosphatehydrogel scaffold	Dropbydropunderstirring	Ultraviolet-visible-near infrared spectrophotometer	-	*In-vitro*	Aggrecan, type II collagen, MMP-3, sulfatedglycosaminoglycans, DNA content., caspase-3 activity.	Confocalfluorescencemicroscope.	Alcian bluestaining, TUNEL staining.

Table 1. Continue

Murab's et al., 2015.	Injectable silk-in-silk integrated scaffold	Lyophilization	Dynamic light scattering, Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer, Rheometer Instrument.	Zwick-Roellservo-hydraulictesting.	Ex-vivo	Cell proliferation kit, aggrecan, type II collagen, SMAD-3, mitogen activated protein kinase-11.	Scanning electronmicroscopy, transmission Electronmicroscopy, Confocal Laser Scanning Microscope.	Safranin-O/alcianbluestaining, Hematoxylin and Eosin.
Willems's et al., 2015.	Thermoreversiblepoly-N-isopropylacrylamidehydrogelscaffold	3D	RheometerInstrument, A colorimetriccompetitive enzymeimmunoassay kit		*In-vitro*&*In-vivo*	Aggrecan, collagentype I and II, disintegrin, matrixmetalloproteinase 13, tissue inhibitor Ofmetalloproteinase 1 inflammation: tumor necrosisfactoralpha, interleukin-1β, interleukin-6, interleukin-10, prostaglandin E synthase 1, prostaglandin E synthase 2,cyclooxygenase 1, and 2, brachyury (T), cytokeratin-8, cytokeratin-18 WNT signaling pathway.	Magneticresonanceimage,	Hematoxylin and Eosin. With picrosirius red/alcian blue, TUNEL staining.

CONCLUSIONS

In parallel with the technological advancements, molecular researches oriented at IVD treatment are coming prominence day by day. Brain and orthopedic surgeons as well as clinical pharmacologists can set up their own studies and make analyzes. Cell based regeneration treatments are promising in the treatment of degenerative discs, which might affect health and socioeconomic conditions negatively.

Conflict of Interest

All authors certify that they, or a member of their immediate family, have no funding or commercial associations (e.g., consultancies, stock ownership, equity interest, patent/ licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

ACKNOWLEDGEMENTS

None.

Ethical Approval

The study was carried out with the approval of Local Ethics Board. Informed consent was

obtained from patients attending the Neurosurgery Clinic for use of their cells.

REFERENCES

Alini M, Roughley PJ, Antoniou J et al. (2002). A biological approach to treating disc degeneration: not for today, but maybe for tomorrow. Europian Spine Journal; 11(2): 215-220.

Best MJ, Buller LT, Falakassa J et al. (2015). Risk Factors for Nonroutine Discharge in Patients Undergoing Spinal Fusion for Intervertebral Disc Disorders. The Iowa orthopaedic J; 35: 147-155.

Bhattacharjee M, Miot S, Gorecka Aet al. (2012). Oriented lamellar silk fibrous scaffolds to drive cartilage matrix orientation: towards annulus fibrosus tissue engineering. ActaBiomaterial; 8(9): 3313-3325.

Bruehlmann SB, Rattner JB, Matyas JR et al. (2002). Regional variations in the cellular matrix of the annulus fibrosus of the intervertebral disc. The Journal of Anatomy; 201: 159-171.

Chan SC, Bürki A, Bonél HM et al. (2013). Papain-induced in vitro disc degeneration model for the study of injectable nucleus pulposus therapy. Spine Journal; 13(3): 273-283.

Cheng YH, Yang SH, Lin FH (2011). Thermosensitive chitosan-gelatin-glycerol phosphate hydrogel as a controlled release system of ferulic acid for nucleus pulposus regeneration. Biomaterials; 32(29): 6953-6961.

Cheng YH, Yang SH, Su WY et al. (2010). Thermosensitive chitosan-gelatin-glycerol phosphate hydrogels as a cell carrier for nucleus pulposus regeneration: an in vitro study. Tissue Engineering Part A; 16(2): 695-703.

Christoph W, Mercedes LR, H Michael et al. (2011). Histological analysis of surgical lumbar intervertebral disc tissue provides evidence for an association between disc degeneration and increased body mass index. BMC research notes; 4: 497.

Ciftci Z, Deniz M, Yilmaz I et al. (2015). In vitro analysis of a novel controlled release system designed for intratympanic administration of N-acetylcysteine: a preliminary report. American journal of otolaryngology; 36(6): 786-793.

Dogan M, Isyar M, Yilmaz I et al. (2016). Are the leading drugs against Staphylococcus aureus really toxic to cartilage. J. Infect. Pub. Health; 9(3): 251-258.

Errington RJ, Puustjarvi K, White IR et al. (1998). Characterisation of cytoplasm-filled processes in cells of the intervertebral disc. The Journal of Anatomy; 192: 369-378.

Feng G, Li L, Liu H et al. (2013). Hypoxia differentially regulates human nucleus pulposus and annulus fibrosus cell extracellular matrix production in 3D scaffolds. Osteoarthritis Cartilage;21(4): 582-588.

Gumustas F, Yilmaz I, Sirin DY et al. (2016). Chondrocyte proliferation, viability and differentiation is declined following administration of methylphenidate utilized for the treatment of attention-deficit/hyperactivity disorder. Human & experimental toxicology; 36(9): 981-992.

Gumustas SA, Isyar M, Topuk S et al. (2016). Systematic evaluaiton of drug-loaded hydrogels for application in osteosarcoma treatment. Current Pharmaceutical Biotechnology; 17(10): 866-872.

Gumustas SA, Yilmaz I, Isyar M et al. (2016). Assessing the negative impact of phenyl alkanoic acid derivative, a frequently prescribed drug for the suppression of pain and inflammation, on the differentiation and proliferation of chondrocytes. Journal of Orthophedic Surgery Research; 11(1): 70.

Guzelant AY, Isyar M, Yilmaz et al. (2017). Are chondrocytes damaged when rheumatologic inflammation is suppressed?. Drug Chemical Toxicology; 40(1): 13-23.

Handley C, Goldschlager T, Oehme D et al. (2015). Mesenchymal stem cell tracking in the intervertebral disc. World journal of stem cells; 7(1): 65-74.

Hanley EN Jr DR, McCulloch JA (1996). Surgical Indication and Techniques International Society for the Study of the Lumbar Spine. The lumbar spine, 2nd ed Philadelphia. PA, WB Saunders: 492–524.

Hunter W (1995). On the structure and diseases of articulating cartilage. Clinical orthopaedics and related research; *42*b: 514-521.

Isyar M, Bilir B, Yilmaz I et al. (2015). Are biological agents toxic to human chondrocytes and osteocytes. Journal of Orthophedic Surgery Research; 10: 118.

Isyar M, Gumustas SA, Yilmaz I et al. (2016). Are we economically efficient enough to increase the potential of in vitro proliferation of osteoblasts by mean of pharmacochemical agents. Open Orthophedic Journal; 10: 420-430.

Isyar M, Yilmaz I, Nusran G et al. (2015). Safety of bioabsorbable implants in vitro. BMC Surgery; 15: 127.

Isyar M, Yilmaz I, Sirin DY et al. (2016). A practical way to prepare primer human chondrocyte culture. Journal of Orthophedia; 13(3): 162-167.

Jalani G, Naccache R, Rosenzweig DH et al. (2016). Photocleavable hydrogel-coated upconverting nanoparticles: A multifunctional the ranostic platform for NIR imaging and on-demand macromolecular delivery. Journal of the American Chemical Society; 138(3): 1078-1083.

Koepsell L, Remund T, Bao J et al. (2011). Tissue engineering of annulus fibrosus using electrospun fibrous scaffolds with aligned polycaprolactone fibers. Journal Biomedical Material Research; 99(4): 564-575.

Kumar D, Gerges I, Tamplenizza M et al. (2014). Three-dimensional hypoxic culture of human mesenchymal stem cells encapsulated in a photocurable, biodegradable polymer hydrogel: a potential injectable cellular product for nucleus pulposus regeneration. ActaBiomaterial; 10(8): 3463-3474.

Li Z, Lang G, Chen X et al. (2016). Polyurethane scaffold with in situ swelling capacity for nucleus pulposus replacement. Biomaterials; 84: 196-209.

Liang CZ, Li H, Tao YQ et al. (2012). Dual delivery for stem cell differentiation using dexamethasone and bFGF in/on polymeric microspheres as a cell carrier for nucleus pulposus regeneration. Journal of Materials Science: Materials in Medicine; 23(4): 1097-1107.

Lipson SJ, Muir H (1981). 1980 Volvo award in basicscience. Proteoglycans in experimental intervertebral disc degeneration. Spine; 6: 194–210.

Murab S, Samal J, Shrivastava A et al. (2015). S. Glucosamine loaded injectable silk-in-silk integrated system modulate mechanical properties in bovine ex-vivo degenerated intervertebral disc model. Biomaterials; 55: 64-83.

Postacchini F, Belocci M, Massobrio M (1984). Morphologic changes in annulus fibrosus during aging: an ultra-structural study in rats. Spine; *9*(6): 596.

Renani HB, Ghorbani M, Beni BH et al. (2012). Determination and comparison of specifics of nucleus pulposus cells of human intervertebral disc in alginate and chitosan-gelatin scaffolds. Advanced Biomedical Research; 1: 81.

Sive JI, Baird P, Jeziorsk M et al. (2002). Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs. Molecular Pathology;55(2): 91-97.

Smith LJ, Gorth DJ, Showalter BL et al. (2014). In vitro characterization of a stem-cell-seeded triple-interpenetrating-network hydrogel for functional regeneration of the nucleus pulposus. Tissue Engineering Part A; 20(13-14): 1841-1849.

Uddin OM, Haque R, Sugrue PA et al. (2015). Cost minimization in treatment of adult degenerative scoloosis. Journal of Neurosurgery: Spine; *23*(6): 798-806.

Xu H, Xu B, Yang Q et al. (2013). Fabrication and analysis of a novel tissue engineered composite biphasic scaffold for annulus fibrosus and nucleus pulposus. Chinese Journal of Reparative and Reconstructive Surgery; 27(4): 475-480.

Yeganegi M, Kandel RA, Santerre JP et al. (2010). Characterization of a biodegradable electrospun polyurethane nanofiber scaffold: Mechanical properties and cytotoxicity. ActaBiomaterial; 6(10): 3847-3855.

Yilmaz I, Akkaya S, Isyar M et al. (2016). Is there a treatment protocol in which platelet-rich plasma is effective. Journal of Orthophedia; 13(4): 316-321.

Yoshiiwa T, Miyazaki M, Notani N et al. (2016). Analysis of the relationship between ligamentum flavum thickening and lumbar segmental instability, disc degeneration, and facet joint osteoarthritis in lumbar spinal stenosis. Asian spine journal; 10(6): 1132-1140.

Zhou X, Tao Y, Wang J et al. (2016). Three-dimensional scaffold of type II collagen promote the differentiation of adipose-derived stem cells into a nucleus pulposus-like phenotype. Journal of Biomedical Material Reserach Article; 104(7): 1687-1693.