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T.C.

ANKARA YILDIRIM BEYAZIT UNIVERSITY

HEALTH SCIENCES INSTITUTE

STUDY OF PREGNANT RAT MATERNAL SERUM EFFECTS ON CHONDROGENESIS IN OSTEOARTHRITIS IN VITRO MODEL

DOCTORAL THESIS

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MUSCULOSKELETAL SYSTEM AND

REGENERATIVE MEDICINE PROGRAM

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Doctoral Thesis

21.09.2020

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BEYAN

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21.09.2020

Ali TORABI

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ÖZET

İn-Vitro Osteoartritik Modelde Hamile Sıçan Maternal Serumu Kondrojenez Üzerine Etkilerinin Araştırılması

Fetomaternal Mikrokimerizm, gebelik sırasında maternal dolaşım içinde fetal hücrelerin bulunması olarak tanımlanır. Çalışma, osteoartritik bir hastadan alınan osteoartritik kondrosit üzerinde kondrojenez yeteneğini ortaya çıkararak, osteoartrit (OA) invitro için olası rejeneratif kapasite rolünü ele aldı.. Bir OA hastasından kondrositin birincil kültürlenmesinden sonra hücreler, 24lük well platede alt kültürlendi ve çalışma için dört farklı gruba ayrıldı. Muayene grubumuz hamile sıçan materyal serumu (PRMS) ile tedavi edilen hücrelerden oluşmaktadır. Karşılaştırma için, normal tam besiyeri dışında hiçbir şey eklemeyerek, hamile olmayan sıçan serumu (NPRS) ekleyerek ve HBBS, kalsiyum ve magnezyum çözeltisi ekleyerek ana, negatif ve pozitif kontrol grupları olarak değerlendirildi. Tüm bağımsız değişkenler aynı anda ve kültür plakalarına toplam ortamın % 10'u kadar eklendi. Aynı ilave miktarı tüm gruplar için eşit yapıldı. Alt kültürleme gününden sonra, hücre ortamı sonraki 10 gün boyunca açıklanan aynı yöntemle tazelenmiştir. 11. günde kuyucuklar sabitlendi ve sırasıyla kondroitin, keratan sülfat, kollajen ve kalsiyum birikimini değerlendirmek için Alician mavisi, Sirius kırmızısı ve Alizarin kırmızısı ile boyandı. Hücresel morfoloji, kondron işlemlerinin uzunluğu, hücre ve kondron sayıları da çalışmada karşılaştırma faktörleri olarak kabul edildi. PRMS ile tedavi edilen grup, çok daha yüksek hücre ve kondron sayıları, daha az kondron işlem uzunluğu ve daha yüksek Sirius kırmızı lekesi gösterdi (P. Değerleri: Kolajen boyama yoğunluğu <0.001, kondron sayısı <0.000, kondron uzunluğu <0.015). Ek olarak, PRMS ile tedavi edilen grupta hücresel ve kondron geometrik morfolojisi (Poligonalite), intraterritoryal ve hücre dışı matrislerde iyi miktar ve dağılım gösteren normal kondrosit benzer şekiller ve daha az kondroptoz olarak daha iyi kalitatif veriler bulundu. Hamile materyal serumu, OA tedavisi için umut verici bir terapötik yol olarak kabul edilme potansiyelini göstermiştir.

Anahtar Kelimeler: Fetomaternal mikrokimerizm, hamile sıçan materyal serumu, kondrosit, kondrons, osteoartrit.

ABSTRACT

Study of Pregnant Rat Maternal Serum Effects on Chondrogenesis in Osteoarthritis In Vitro Model

Fetomaternal Microchimerism defined as the presence of fetal cells inside the maternal circulation during pregnancy. Our study addressed its possible regenerative capacity role for osteoarthritis (OA) invitro by challenging its chondrogenesis ability on the osteoarthritic chondrocyte that harvested from an osteoarthritic patient. After primary culturing the chondrocyte from an OA patient, cells were subcultured on a 24-well plate and divided into four different groups for study. Our examination group composed of the cells treated with pregnant rat maternal serum (PRMS). For comparison, we considered a main, negative, and positive control groups by not adding anything extra than normal complete medium, adding non-pregnant rat serum (NPRS), and adding Hanks' Balanced Salt with calcium and magnesium solution respectively. All of the independent variabilities added at the same time and for 10% of total media in culture plates. We consider the same add-on amount for the main control group from the normal complete medium as well. After the subculturing day, cell media were refreshed with the same explained method for the next 10 days. On the day 11th wells were fixed and stained with Alician blue, Sirius Red, and Alizarin red for evaluating chondroitin and keratan sulfate, collagen, and calcium deposition respectively. The cellular morphology, chondron processes' length, cell and chondron counts were also considered as comparison factors in our study. The group treated with PRMS shown much higher cell and chondron counts, less chondron process length, and higher Sirius red stain (P.Values: Collagen staining intensity<0.001, chondron count<0.000, chondron length<0.015). In addition, we found better qualitative data in PRMS treated group as cellular and chondron geometric morphology (Polygonality), normal chondrocyte similar shapes with good amount and distribution in the intraterritorial and extracellular matrixes as well as less chondroptosis. Pregnant maternal serum shown its potential to being considered as a promising therapeutic way for OA treatment.

Keywords: Chondrocyte, chondrons, fetomaternal microchimerism, osteoarthritis, pregnant rat maternal serum

ABBREVIATIONS

ACI : Autologous Chondrocyte Implantation

DMEM : Dulbecco’s Modified Eagle Medium

FBS : Fetal Bovine Serum

GAG : Glycosaminoglycans

HBSS: Hanks' Balanced Salt Solution

ICRS : International Cartilage Repair Society

IL-1β : Interleukin 1 beta

IL37 : Interleukin 37

IL6 : Interleukin 6

IL8 : Interleukin 8

MMP-3 : Matrix Metalloproteinase-3

MSC : Mesenchymal Stem Cells

NPRS: Non-Pregnant Rat Serum

NSAID : Non-steroidal anti-inflammatory drugs

OA : Osteoarthritis

OARSI: Osteoarthritis Research Society International

OAS-CM:Osteoarthritic Synovium-Conditioned Medium

PDGF-BB : Platelet-derived growth factor BB

PRMS: Pregnant Rat Maternal Serum

TGF-beta 1 : Transforming growth factor beta1

TNF-alpha : Tumour Necrosis Factor alpha

UCB : Umbilical Cord Blood

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1.INTRODUCTION

Osteoarthritis (OA) is chronic damage of articular cartilage. It is a well-known condition and one of most destructive condition in all world (1). Referred to a recent data from the National-Health-Interview Survey, its about 14 million of population in united States who suffering from symptomatic osteoarthritic knee which more than half of them are over 65 years old (2).Many of cohort and community-based studies have also measured the prevalence of OA in various communities. Results shows high prevalence rate for the disease in different parts of the world and despite of kilometers of geographic distance in-between them, almost the ratios are close and this shows the genetically issues would not be only the factor and somehow environmental and lifestyle factors having the crucial role in the prevalence of this disease:

1. South America:
    a. In the study made in Rosario, Argentina estimated prevalence was 4% (3)
    b. In the another study which made in Chihuahua, Mexico estimated prevalence was 6.6% (4).
2. Asia:
    a. In the study which made in south Korea, the prevalence of radiographic knee osteoarthritis was up to 21.1% in males and up to 43.8% in females. Also estimated symptomatic knee osteoarthritis prevalence in this study was 4.4% for male and 19.2% for female population (5).
    b. In the another studies which made in India overall prevalence of the knee osteoarthritis was estimated up to 28.7% (6).

c. In an interesting study done in japan prevalence of the osteoarthritis in hand were estimated up to 89.9% and 92.3% in man and women respectively, radiographic methodology with Kellgren-Lawrence scale (7).

3. Middle east :

a. In a study made in Iran Osteoarthritis prevalence estimated 16.9% (8).

Impact of the such a disease with this high ratio of global prevalence should be terrifying on global health level so is completely worded for being selected as one of the most negative influencer of the population and nations' life quality and economy respectively. There are many studies showing the long life living of the Osteoarthritic patients with disability which notice how important is this disease in bringing the chronic disability aspect (9, 10). People with OA also shows more susceptibility of developing mental disorder as depression symptoms (11). OA was also associated with greater odds of suicidal ideation (12). There are some other evidence that OA influence negatively in other mental and physical disease as; memory loss mediated by sleep and mood impairment (13) and cardiovascular events (14-16). As mentioned earlier OA as a chronic disease with increased chance of acute events being added on its chronicity such as different kinds of fractures, has also financial cost load on the patient individually and health system nationally. Based on statistical researches, OA showed the higher hospitalization risk alongside with higher expends (17, 18). OA average cost in Canada increased $577 to $811 per patient/year between 2003-2010(19) In America, Total annual average for each patient reported between $1,442 to $21,335 (adjusted to 2015 US$ equivalent) (20).

This entrance in prevalence and impacts of OA disease completely clears the crucial cause of efforts which have been made by scientists and clinicians to find the better ways of preventing OA or at least control the disease and preventing appearance of its sequel or even cure it. Considering the disease pathophysiological facts, damaged cartilage has limited regenerative capacity so intervention will be the crucial part of the approach to it. In this manner we may divide this efforts into:

- Conservative clinical ways: which ofcource is not the definite cure in the disease but may subsides the symptoms and decrease the disease worsening clinically or even on pathophysiological prospects. Rehabilitation and physiotherapy, ultrasound therapy, varies of drugs which is not given for curative reasons but only for symptom therapy and boost the life style (NSAIDs, Glucocorticosteroids,…)

- Surgical non-regenerative way : this way of approach to the OA usually being indicated in higher grades of the disease. Despite its efficacy in preventing lots of early and late sequels of the disease and almost complete removal of the most symptoms and signs, this way is also should not be categorized as cure way in the disease.

- Regenerative ways: The one and only way that can give a chance of the cure in OA patients coincide with subsiding the signs and symptoms are the ways that being covered by regenerative medicine. Some of these strategies are, bone marrow stimulating, osteochondral autograft, osteochondral allograft, autologous chondrocyte implantation (ACI), DeNovo-NT (Zimmer) and utilizing stem cells therapies (21-25).

There are many invitro and invivo studies are made in this prospect and some of them leads to the good results. For better understanding the regenerative approaches we may enter separately as INVIVO and INVITRO studies.

INVITRO:

Because of feasibility, availability and less ethical issues of the invitro studies, these kinds of studies are always being selected by scientists and yet there are lots of dramatic improvements has been done by regenerative scientist in this category.

a. In a study Interlukin-37 somehow reversed IL1β- and OA synovium-conditioned medium-impaired cartilage formation of MSC in vitro. IL37 showed enhancement of sGAG amount in IL1β-and OAS-CM treated pellets at least for 1.5 fold. Gene expression analysis also revealed reduction in IL1B, IL6 and IL8

gene expression as well as blocking MMP3 and protein expression. There were not hypertrophy marker expression seen in this study by applying IL37 (26).

b. In another study bone marrow derived progenitor cells shows the invitro capability of chondrogenesis which have been showed that MSC's are good sources of cell to work on (27, 28).

c. In a study chondrogenesis capacity of utilized stem cells gotten from Wharton jelly were challenged which ended to positive results (29).

INVIVO-INVITRO:

a. In a study Bone Marrow Mesenchymal proginator cells were treated with two growth factor of TGF-beta and PDGF-BB. Invitro and Invivo approach were considered in the study. Both of these factors increased proliferation amount but decreased alkaline phosphatase activity of MSC. After trypsinization of MSC, cells were splitted into two parts. Invitro approach were made by seeding cells in osteogenic medium, whereas another part seeded into porous calcium phosphate ceramics and implanted subcutaneously in syngeneic rats for invivo approach. PDGF-pretreated MSC didn't show any difference in both approaches, but TGF-beta1 pretreated cells blocked osteochondrogenic behavior of MSC invitro but they showed the osteoblastic and chondrogenic ability invivo (30).

b. In an another study ability of harvesting MSC from synovial membrane tissue and its proliferation and differentiation capability were studied which shows the high potential of this cells to be a source for cell therapy o another cell based studies (31).

c. One of the very feasible MSC sources which also open its way into clinic and getting popular between orthopedic surgeons is the deriving MSC cells from adipocyte tissues which invitro studies shows that they have a good capability in chondrogenesis (32).

INVIVO:

1. Animal studies:

    a. In a study, bone marrow and periosteum derived mesenchymal cells were seeded in collagen gel. Healing of osteochondral structure was observed by this approach (33). Some other stem cell implanting approaches also showed the good results as well (34-40). Some studies in this manner even shows the capability of the allogenic source of MSC for induction of chondrogenesis activity (41).

    b. In another study human amniotic MSC were shown to be have chondrogenetic potential (42).

2. Clinical Studies:

    a. Currently, MSCs are used in the clinic either by direct placement in cartilage defects or indirectly by microdrilling approaches (43).

    b. There were some studies shows that the peripheral blood derived stem cells also shows the good results in inducing chondrogenesis (44-46).

As far as we mentioned until now most of the studies specially those which resulted to the good chondrogenesis capability were generally based on cell therapy only. There are some studies which demonstrated that mixture use of cells alongside with growth factor on scaffold increased articular cartilage healing(40). Healing enhancement by adding growth factors effect directly or by gene transfer showed to be promising also in another studies(47-50). Using of growth factor as the main inducer of the repair is something less been suggested or confirmed yet but effect of them as an enhancer for chondrogenesis is a considerable and crucial point which is both proved invitro and invivo. One of the growth factor usage which got fix its own place in the clinic as a popular repair (or at least semi-repair) method of OA disease is usage of platelet rich plasma (PRP). Growth factors from platelets are assumed to improve the outcomes in OA knee and even after total knee arthroplasty (TKA), however the

efficacy ratio of this method is still controversial between scientists but at least almost all of them unanimously agree with short term efficacy of it in OA management (51-55).

At the other hand on many of the related studies in regenerative field varies of scaffold had been used for seeding of the cells. Collagen (33, 39), hyaluronic acid (36, 56), polyglycolic acid (57), poly-(lactic-co-glycolic) acid (40, 58), and silk fibroin (59) usage as scaffold were introduced in lots of studies.

Pre or post differentiation strategy for implanting has been researched by scientists. Some studies showed that predifferentiated and undifferentiated MSCs usage will not lead to any significant histologic and biochemical different results (60, 61).

Unfortunately, the cartilage repair with these strategies cartilage repair occurs not always satisfactory, due to variability in the amount of newly formed cartilage and the formation of fibrocartilage, which is structurally inferior to hyaline cartilage (62-64). Inflammatory pathophysiology of the damage joint has been assumed as cause of these unacceptable results (65). Chronic injured knee synovial fluid extract, showed to have suppressive effect on MSC and reduce cartilage healing (66). This finding also approved as decreasing of GAG content in redifferentiating chondrocyte (67). Next to synovial fluid, also conditioned medium obtained from OA synovium (OAS-CM) blocked chondrogenic differentiation of MSCs (68). Hence, the real capability of the each regenerative approach is worth to being evaluated under the mentioned obstacles either in vivo as animal/clinical studies or in vitro studies by simulating the similar medium and environment as it is when we have osteoarthritis.

2. GENERAL INFORMATION

Hypothesis: Pregnant rat maternal serum can induce the chondrogenesis in invitro osteoarthritic model.

Hypothesis description: We challenge the chondrogenesis capacity of the pregnant rat maternal serum in primary chondrocyte culture from an osteoarthritic patient's knee articular cartilage in terms of induction of proliferation and also the extracellular and intra-territorial matrix production ability.

General idea and aim of the study*:* We assume that the pregnant rat maternal serum contains the fetomaternal microchimeric chondrogenesis inducer factors which can enhance the proliferation and matrix protein production in the chondrogenesis manner. To prove this we applied the serum of pregnant rat into the primary cultured OA-chondrocytes invitro from the first day of subculture, And observe the chondrocyte behavior in our groups.

It’s totally worth to say despite of less efficacy of the growth factor to being use as main inducer of the chondral repair compare to cell therapy or combined cell+growthfactor (growth factor as the enhancer) therapy; feasibility, availability, easier application, lower cost and too much lower risk and ethical issues of the growth factor only methods makes these methods promising clinically. Easy entrance of the PRP method into the clinic may be results of the mentioned fact. Hence, finding the new growth factor method which has much more efficacy on chondral repair would be something dramatically phenomenal which can be use both as enhancer in combined cell therapy and directly in clinical induction therapy. So in this project we wants to work on the new factors which may results to chondrogenesis. Our study is based to the phenomenon called fetomaternal Microchimerism.

2.1. Definitions:

2.1.1. Microchimerism Definition:

Microchimerism has been explained as presence of the cells or any other material related to one genetically distinct individual inside other individual. These trafficking of biologic substance can be in bi-directional way. This phenomena may happens physiologically as in pregnancy, or iatrogenic after organ transplantation or blood transfusion. The migrated cells has ability to remain in host body for decades (69). Presence of microchimeric material has been studied widely in multiple pregnancy (70), blood transfusion (71), solid organ transplant (72), and bidirectional trafficking during pregnancy (73).

2.1.2. Microchimerism History:

Georg Schmorl was the person who described this phenomena for the first time in 1893. Appearance of trophoblastic originated cells were inspected by him which he postulated these cells as fetal originated cells (69). Other studies also confirmed Schmorl hypothesis as appearance of the trophoblastic characterized cell in maternal blood circulation (74, 75). Some other type of cells also reported from maternal circulation later, such as erythrocytes, nucleated erythrocytes (76, 77), leukocytes (78), hematopoietic stem cells (HSC) (79) and mesenchymal stem cells (MSC) (80). Mentioned cells so called as pregnancy-associated progenitor cells (PAPCs) as they may have the progenitor behavior ability (Khosrotehrani and Bianchi 2005). Microchimerism in term of fetus and mother relationship has been called FetoMaternalMicrochimerism (FMC) (81, 82). Scientists worked on different areas of this phenomena, which intact cells (83), cell-free DNA and messenger RNA sequences (84), such as plasma and serum (85), cerebrospinal fluid (86), and amniotic fluid (87) were some of them.

2.1.3. Fetomaternal Microchimerism:

Placental transmigration of microchimeric cells: Fetal cells during pregnancy has been assumed to migrate toward the mother circulation via placenta. Some earlier studies were suggesting that fetomaternal cell migration starts from early of gestation,

and its amount will increase by gestational-age (88). Some other studies as Rei Sunami et al. studies revealed earlier entrance of these cells (89). Monitoring of women who got pregnant by invitro fertilization proved that fetus originated DNA can be appeared in mother serum from 7th week of gestation (90).

2.1.4 Persistency of Fetomaternal Microchimerism:

Persistency of microchimeric cells: Fetomaternal migrated cells from fetus toward mother during pregnancy, founded to be persisted even years after delivery (69). In another study by Antoni Bayes-Genis and his colleagues, the hearts from non-pregnant women with history of 2 and 3 delivary respectively were studied after their heart transplantation due to idiopathic dilated cardiomyopathy. Non of the cases had history of blood transfusion, autoimmune disease or peripartum cardiomyopathy. Both of the cases at least delivered a male baby and the history of labor and delivery were uneventful for both of them. This study showed the presence of the male cells in heart speciemens of both cases, also its revealed that under the appropriate signaling these cells can differentiate to cardiomyocytes as well. Other studies also confirmed the persistency of these cells in mother's body (91-95).

2.1.5. Microchimeric Cell Residency:

Presence of fetomaternal microchimeric cells in varies of tissues: Fetal microchimeric cells were found in varies organs and tissue types (91, 93, 94, 96-98). Organ such as, liver (99), lung (100), thyroid (101) and skin (102) have been reported as site of these cells migration. However, these cells have been also reported in healthy cases (91, 99-103). Studies also done to show this variability and persistency in animal models, some as short term study and few as long term (104).

2.1.6. Microchimeric Cells Populations:

Fetomaternal microchimeric cell population frequency: The study by Hamada et al, population of fetal cells increased 10 times at term compare to early pregnancy. Population of these cell shown to be increased from 0.0035% in 2nd trimester to 0.008%

in 3rd, Similar result about frequency of cell-free fetal DNA also suggested by others (90, 105, 106).

2.1.7. Varients of Fetomaternal Microchimeric Cells in Maternal Blood:

Fetomaternal microchimeric cell variations: Fetus blood circulation showed to have varies of progenitor type of cells, which may take a part in fetomaternal Microchimerism as well, although the amount of these cells are decreasing by gestational age progression (107).

2.1.8. Signaling in Fetomaternal Microchimerism:

Signaling in fetomaternal microchimeric cells: in the study done by Rey Sunami et.al, they saw fetomaternal microchimeric cells only in bone marrow if no injury applied to any organ of mother during the pregnancy prior to 60th day postnatal hysterectomy. But when they created injury models by streptozotocin injection and supplying arteries ligation to produce infarction in related organs; they have seen the population of the fetomaternal microchimeric cell only in injured area (89). From this study and some other similar studies (108, 109), we hypothesized that migrating and locating of these cells should be highly stimulated by acute signaling process as injury to make a signaling like affect in purpose of taxing/trafficking cell to injured area.

2.1.9. Fetomaternal Microchimerism Differentiation Capacity:

Fetal microchimeric cells multilineage differentiation capability: By observation of mature fetus originated cell in varies of maternal tissues and organs, scientists postulated that these cells have the ability to differentiate into variant kinds of tissues (89). Hepatocytes (110-112), renal cells (111), neuronal cells (113, 114), cardiomyocytes (115, 116), intestinal epithelium (96) and endothelial cells (117) and Thyrocytes (118) are some examples of mentioned tissues.

2.1.10. Fetomaternal Microchimeric cells and Stem cell nomenclature:

Capability of fetomaternal microchimeric cells to being named as stem cell: Their wide differentiation capacity to several kinds of tissues and their persistency feature make these cells worded to being considered as stem cells as well.

2.1.11. Immunologic aspect of the Fetomaternal Microchimeric Cells:

Higher immune capability of this source: There are studies show that using perinatal sources (cord blood, umbilical cord, amnion, and chorion,…) of stem cells and their derivatives as allogenic sources of therapy has a potential to modulate Invitro immune response (119). Hence these kind of sources seems to be promising in allogenic usage of invivo studies and in clinic.

2.2. Study Design Principles

1) Aim of the study: Is to challenge the chondrogenesis capability of 'fetomaternal Microchimerism'.

2) Study base method: we design our study as invitro study with one main control group, one negative control group, one positive control group and one experimental group.

3) Our main independent variable would be the 'Pregnant Rat Maternal Serum (PRMS)' which would be evaluated in the experiment.

4) As we mentioned in main introduction part, evaluating of one subject capability for chondrogenesis is good to be done in the OA simulated medium which can resemble the disease situation. Positive results from such a study design would be highly guaranty the future clinical study of the regarded subject. In this manner we decided to primary culture chondrocyte cells gotten from Osteoarthritic patient who is undergoes to total knee replacement arthroplasty surgery, and use obtained chondrocytes as our dependent variable base to see effect of PRMS on them in our experimental group. These cell is obtained from distal femoral condyles and proximal tibial facet cartilage of the patients which routinely taken during total knee

arthroplasty surgery. Similar manner of Osteoarthritic cells usage have been widely worked in many of OA disease researches (120-124).

5) Our main control group was the normal culture of the HC-OA without adding any extra independent variable to the culture. And it was subcultured and monitored by changing the growth medium only.

6) Our positive control group was the culture of chondrocytes with adding hanks balanced salt solution(HBSS) during subculture which expected to result in cell shrinkage due to the decreasing in the PH as HBSS known to be not appropriate for incubation in CO2 incubators (125).

7) Our negative control group designed as adding of the Non-Pregnant Rat Serum(NPRS) to the OA-chondrocyte sub-culture which expected to have not any effect in comparison with main control groups.

8) Finally our main experimental group, which adjusted as adding of the PRMS to the osteoarthritic subculture.

9) All the independent variables mentioned here are designed to add from first step of the subculture by ratio of 10% to the basic complete media needed for each cell passage.

2.3. Analysis and Comparison Between the Groups Principles

INVIVO osteoarthritic chondrocyte behavior has been categorized by Sandel (126):

- Proliferation and cell death (apoptosis)
- Changes in synthetic activity
- Degradation
- Phenotypic modulation of the articular chondrocytes:

Fibroblastic phenotyping or dedifferentiation were not reported to be as an important factor invivo. Primitive phenotype has been described as IIA and III precollagens and proteolytic enzymes synthesis. Apoptosis showed to happen in calcified cartilage and Clustering reported as proliferation sign of invivo osteoarthritic chondrocyte behavior.

- formation of osteophytes

Sandel also categorize the phenotypical behavior modulation of the chondrocyte after being at the biological stress. Figure 2.1. is beautifully showing this behavior.

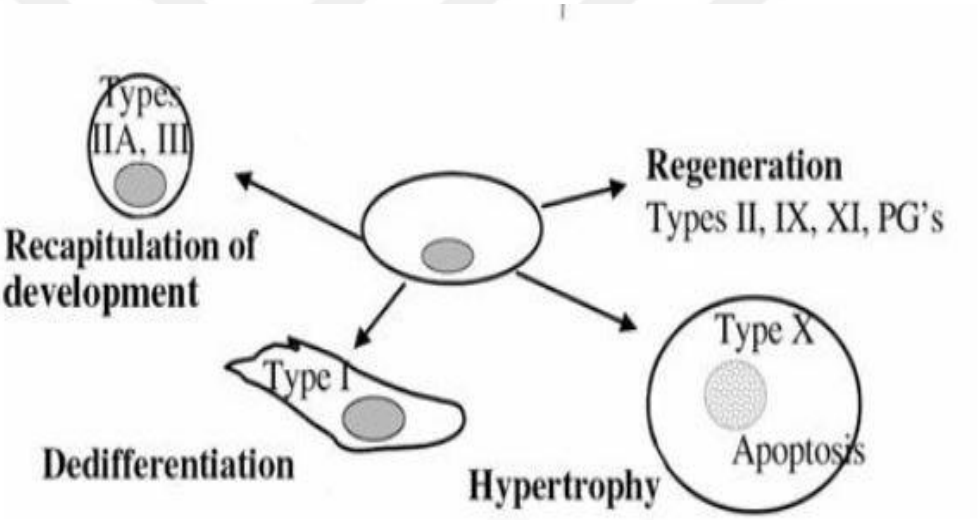


Figure 2.1. Phenotypic behavior of the chondrocyte after being in stress (126).

Chondrocyte development, dedifferentiation, hypertrophy or regeneration of mature cartilage reported to be a sign of chondrocyte activity (126). Figure 2.2. shows the cellular biology response summery invivo of OA patients.

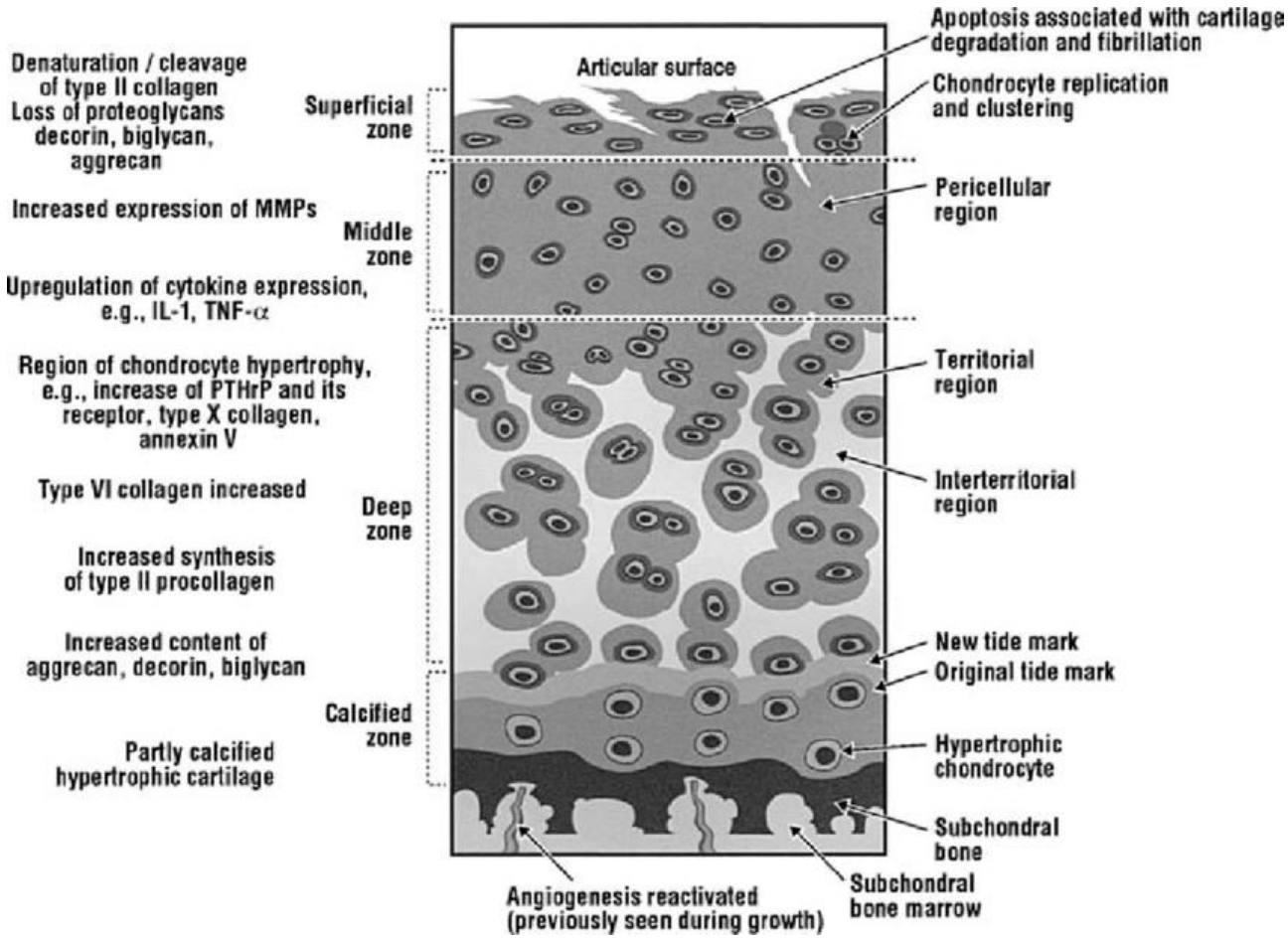


Figure 2.2. Summary of the changes occur invivo of OA patient (127).

Scientist also published histopathological grading of the OA by these changes. The study done by Pritzker et.al is truly one of the complete evolution of the OA histopathology yet and their grading system also have been accepted by ICRS(International Cartilage Repair Society) and OARSI(Osteoarthritis Research Society International). Yellow marked sections is marked by us to show the cellular based factors in Pritzker grading system which is more notable for our study since part of our study is based on cellular morphology (Figure 2.3).

A cartilage histopathology grade assessment—grading methodology

Grade (key feature)	Associated criteria (tissue reaction)
Grade 0: surface intact, cartilage morphology intact	Matrix: normal architecture Cells: intact, appropriate orientation
Grade 1: surface intact	Matrix: superficial zone intact, oedema and/or superficial fibrillation (abrasion), focal superficial matrix condensation Cells: death, proliferation (clusters), hypertrophy, superficial zone Reaction must be more than superficial fibrillation only
Grade 2: surface discontinuity	As above +Matrix discontinuity at superficial zone (deep fibrillation) ±Cationic stain matrix depletion (Safranin O or Toluidine Blue) upper 1/3 of cartilage ±Focal perichondronal increased stain (mid zone) ±Disorientation of chondron columns Cells: death, proliferation (clusters), hypertrophy
Grade 3: vertical fissures (clefts)	As above Matrix vertical fissures into mid zone, branched fissures ±Cationic stain depletion (Safranin O or Toluidine Blue) into lower 2/3 of cartilage (deep zone) ±New collagen formation (polarized light microscopy, Picro Sirius Red stain) Cells: death, regeneration (clusters), hypertrophy, cartilage domains adjacent to fissures
Grade 4: erosion	Cartilage matrix loss: delamination of superficial layer, mid layer cyst formation Excavation: matrix loss superficial layer and mid zone
Grade 5: denudation	Surface: sclerotic bone or reparative tissue including fibrocartilage within denuded surface. Microfracture with repair limited to bone surface
Grade 6: deformation	Bone remodelling (more than osteophyte formation only). Includes: microfracture with fibrocartilaginous and osseous repair extending above the previous surface

Figure 2.3. Grading system (3) - Note the highlighted texts which refers to cell changes.

Morphology behavior pattern of the chondrocyte invitro have been also considered by some scientist in varies of studies. Musumeci et.al design the study based on invitro morphological pattern of chondrocyte culture and show the apoptotical pattern of cells invitro beside fibroblastic morphological changes occurring by time of culture and number of the passages passed in osteoarthritic chondrocytes (128). This morphological behavior invitro also had been shown by some other studies (129-131). All the groups also have shown the changing of the normal polygonal healthy structure of the chondrocytes to elongated Osteoarthritic (fibroblastization, dedifferentiation) structure with loosing of the cytoplasm and nucleus condensation which results to having smaller chondron size. Increasing the cell-to-cell contact and higher cell density can be another good point of comparison which is belongs to the fibroblast like chondrocytes (132, 133).

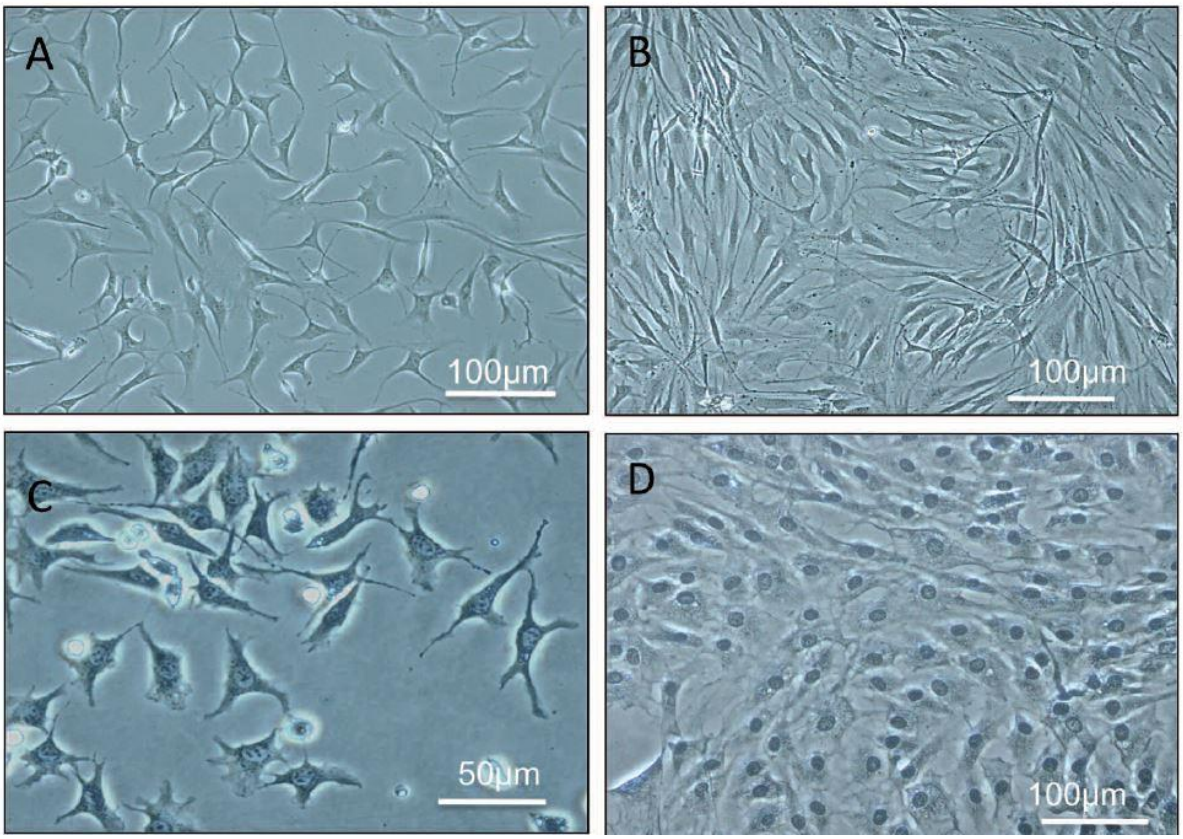


Figure 2.4. Comparison of the normal chondrocyte and OA chondrocyte in second day & 7th day of culture (5) : A.OA chondrocyte in second day of culture; B.OA chondrocyte in 7th day of culture; C. Healthy chondrocyte in second day of culture; D. Healthy chondrocyte at 7th day of culture.

Another variable that we can observe in morphologic change of the chondrocytes is the presence of the chondroptosis. Endoplasmic reticulum and Golgi apparatus increased initially in Chondroptosis which is indicator of protein synthesis enhancement in the cell. ER membrane fragment the cytoplasm and form the areas which organelles and cytoplasm should be digested. These findings are not similar of what we have in normal apoptosis. After demolition happened in autophagic vacuoles, remaining structure blebbed into lacunae. After an complete destruction, empty lacunae will be formed (134).

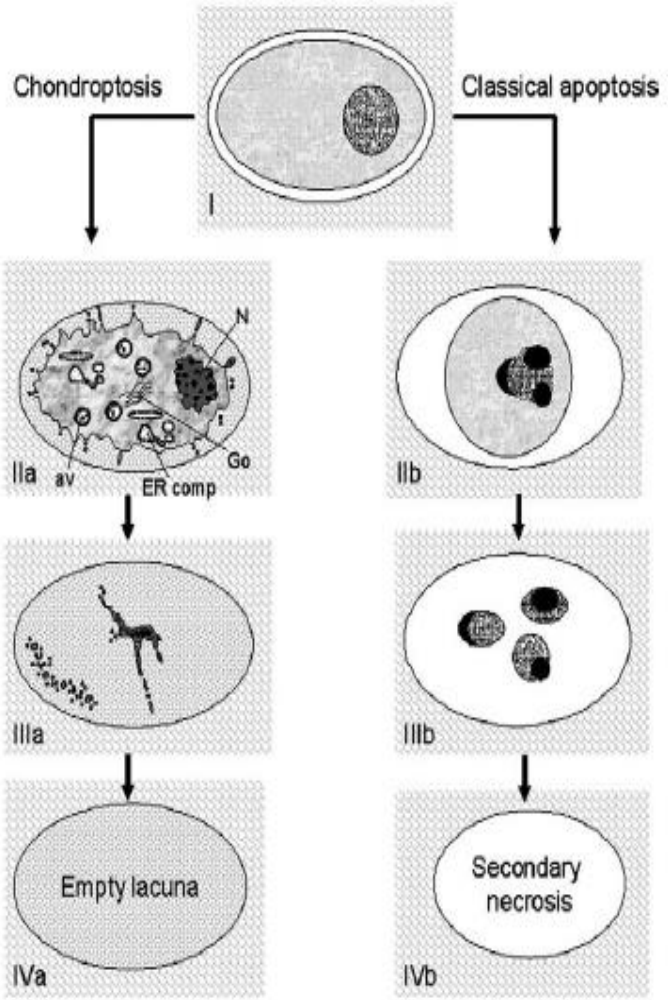


Figure 2.5. Chondroptosis vs classical apoptosis (134).

Chondron remodeling is what we see usually in osteoarthritic chondrocytes. Swelling of pre-cellular microenvironment, initiation of cell division and migration, proliferation alongside modified synthesis are the special chondron remodeling process to form the clusters. Here is the Ross' classification in chondron remodeling (135):

I. Rounded cells in oval or round conformation.

II. Concentric layers of cells densely packed together.

III. A layer of flattened interleaved cells around the outside of the cluster with few cells in the middle.

IV. Tightly packed cells, with membrane boundaries difficult to discern.

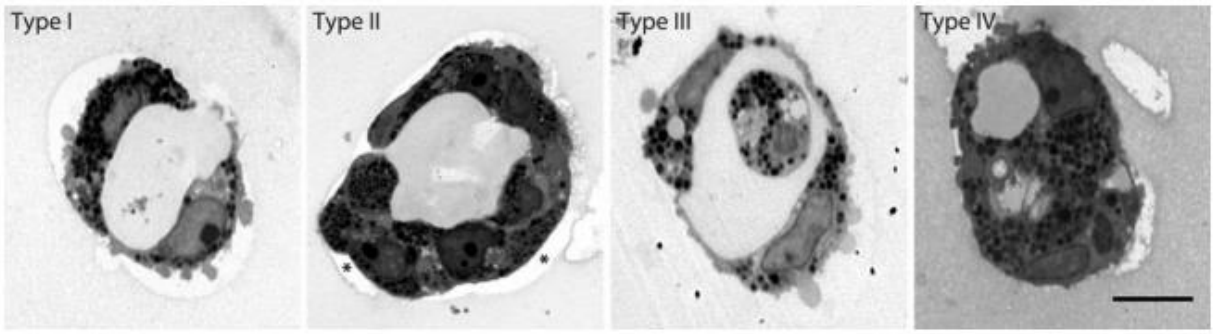


Figure 2.6. Cluster types, Ross's Classification (135).

High quantity of clusters can be an indicator of OA cartilages in most of histologic analysis. Cell proliferation described as the main cause of clustering which can be seen in OA patients (136).

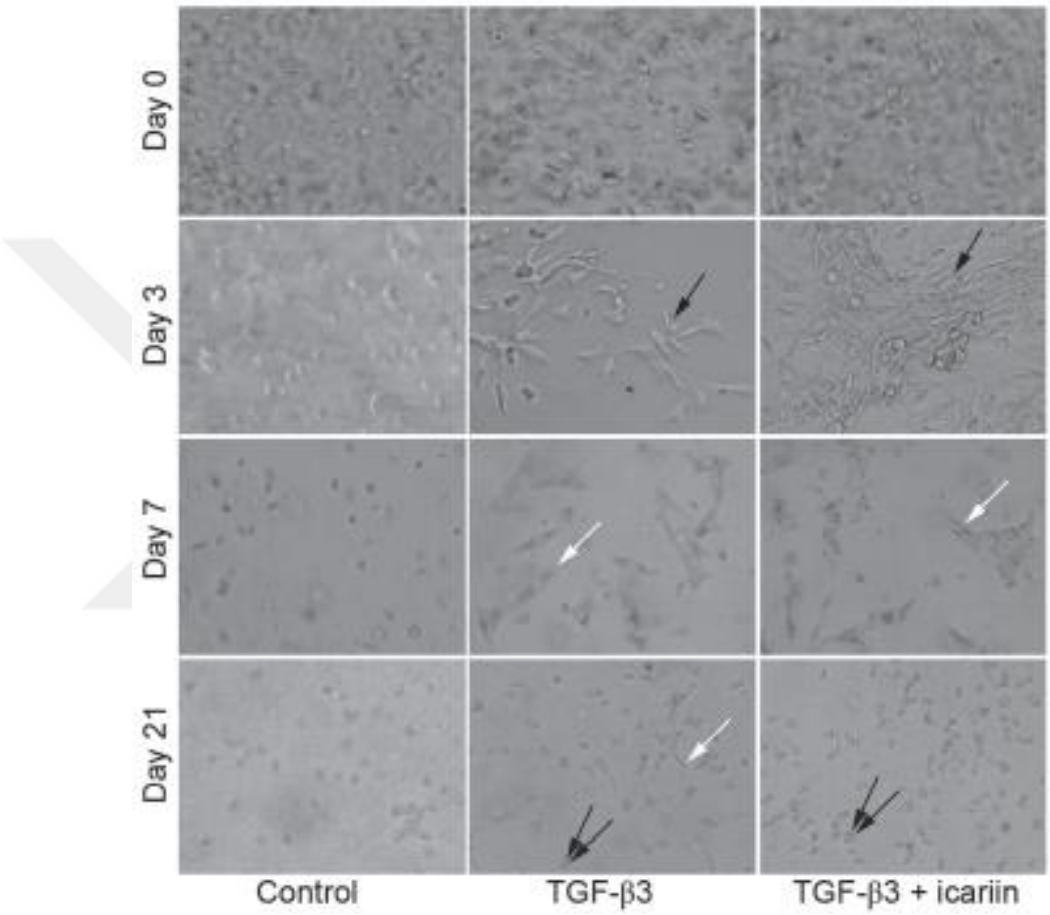


Figure 2.7. Chondrogenic differentiation and cell morphologic comparison. Black arrows indicate cell-cell contacts with a clustered morphology, white arrows indicate fibroblastic spread morphology and double black arrows indicate chondrocyte-like rounded morphology. Magnification, x100. TGF-β3, transforming growth factor-β3 (133).

As we have more osteoartheritic morphology in histological speciemns of the OA patients in deeper zones, the migration of the chondrocytes to this are may reflect the negative morphologic changes as well. Higher amount of golgi apparatus, lipid and protein targeting to provide the energy and the signaling needed for this migration has been showed in literatures. In addtion presence of the primary cilia and filopodia has

been seen in near clusters. Changing in the cytoskletal structures also has been proved in some studies, presence of the vimentin which mentioned earlier as well and also more intrestingly transmission of the actin filament to the α-smooth muscle actin mediates the contractile behavior of cells (136, 137). Length of Cytoplasmic processes generally have been considered as the de-differentiation versus normal chondrocyte morphology slope in some articles (133).

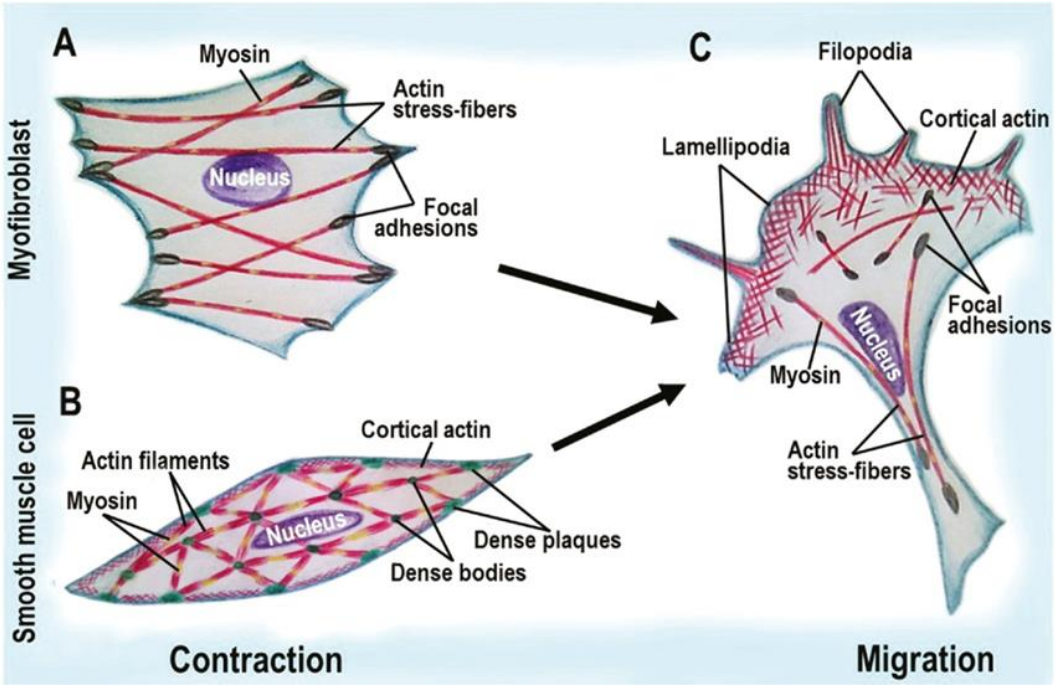


Figure 2.8. Chematic diagram illustrating contraction vs. migration phenotype in fibroblasts and smooth muscle cells.(A) contractile fibroblasts, myofibroblasts, are characterized by the formation of myosin-containing actin contractile stress fibers. (B) in contracting smooth muscle cells, actin assembles into myosin-containing actin contractile apparatus and cortical actin network. (C) migration of both fibroblasts and smooth muscle cells is accompanied by a similar reorganization of actin microfilament system, including lamellipodia and filopodia formation (138).

Triglyceride and sterol esters covered by phospholipids droplets structures can be seen in most of cell types. Their size, location and contents are changing during the cell life cycle. Fat droplets has plays role in cellular metabolism, inflammation, immunity and signaling pathway of the cell (139). As we mentioned earlier, migration needed, cytoskeleton changes, inflammatory process and intensive signaling needed in OA chondrocytes make worth to say that increasing the intracellular lipids may reflect remodeling of OA-chondrocytes from their original more stable chondrocytes form. However, it can be the result of differentiation from its progenitor cell (140). So we may summarize this as if we trying to differentiate a progenitor cell as MSC to form any daughter cell forms, inspection high amount of fat droplet is normal and can be assessed as positive indicator factor in transition to target cells, but presence of them while we culture adult cells as chondrocytes would be the inflammation or de-differentiation process. In other study made by COLLINS and his colleagues, More intracellular lipids reported in specimens gotten from aged individuals than younger ones. Additionally they report that the site of the cartilage specimen effects amount of the lipid droplets as central portion of costal and bronchial cartilage has more lipid droplets than periphery (note the fact that generally peripheral site of the cartilages reported as having much more healthier cells, less inflammable and less fragile than central part (141) alongside with less droplet in articular and subperichondrial healthy cartilages (142).

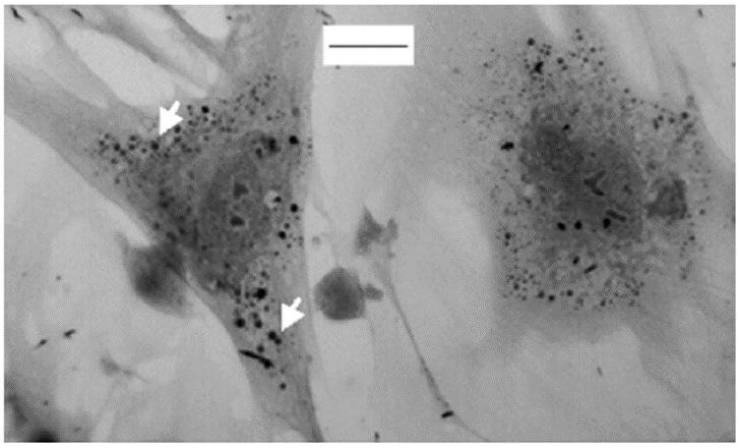


Figure 2.9. Tiny intracytoplasmic lipid droplets (arrowheads) present in UCB-derived MSCs under standard differentiation conditions.Magnification: 31000. The bar indicates 20 lm. (140).

Hence, refer to what mentioned above we decided to compare the groups based on 3 factors:

I. Cell quantity in constant microscopic magnification field: cell counting have been done in inverted microscopy after imaging. The intracellular space also considered as growth phase indirect evaluation of the cells.

II. Cellular morphology : The presence of chondrons with long processes (Chondron length), high volume, and clustering suggests important negative changes that we usually see in osteoarthritic cell morphologies (143). Also as we already mentioned other factors will be noted in analysis of morphology: Intercellular interactions, chondroptosis, lipid droplets and geometric attributes.

III. Cell lines were stained with Alician Blue dye solved with HCL for staining strongly sulfated mucosubstances such as chondroitin and keratan Sulphate. Sirius Red dye is used for staining collagen. And Alizarin Red dye is Used for monitor calcium contents in case of any ossification process happen.

Statistics Analysis Method : Chondron process (filopodia) length, Chondron Count and sirius red staining color value level (Calculated by chondron mean histogram color value) after being calculated by NIH Image J software were analyzed statistically by One-way ANOVA test. Since we duplicate the experiment while passage it in 24 well plate, we divide 24 well plate in two experiment at the same time as 12 associated to one and 12 to other same time experiments. The reason for this is to reduce the chance of error as much as possible. Hence, the mean value of each statistical quantitative analysis factors between two general experiments have been included in ANOVA test. Data analyzed by this test, and p.value less than 0.05 considered as significant result.

3. MATERIAL AND METHOD

3.1. OA-Chondrocyte Primary Culture

3.1.1. Material

Transfer Media : Prepared with DMEM contains 5% of Penicillin-Streptomycin (10,000 U/ml). Penicillin-Streptomycin solution step thawed first in +4, Then sterile filtered in 0.20 micrometer strain filters. Complete Media : Prepared with DMEM contains 1% of Penicillin-Streptomycin and 10% of FBS. FBS step thawed in to +37, each 10 minutes mixed gently during unthawing process. FBS as like as Penicillin-Streptomycin also sterile filtered in 0.20 micrometer strain filters. digesting Media without collagenase: Prepared with DMEM + 5% FBS. Preparing digesting media without collagenase prior to adding collagenase at digesting step make the steps easier to make and reduce the chance of error which we recommend it. Mechanical harvesting instruments: Scalpel blade and Rongeur instrument have been used to cut, dice and smash the tissue samples precisely. All the instruments have been autoclaved prior to use. Collagenase Type II enzyme Mixture : Collagenase type II (Gibco, Cat.no.17101-015) reconstituted with HBSS solution to bring the solution to 100 U/micro liter(1000X stock solution) and aliquoted the in sterile eppendorf and kept in -20 for further uses. stocked eppendorf has been removed and unthawed in ice in the room temperature prior to use and further HBSS added to reach 0.5% W/V (50-200 U/ml) concentration as manufacturer recommended. Prepared solution filter sterilized with 0.20 micrometer strain filters and kept in refrigerator for the short time until the use. Transfer Equipment: Cool box has been used.

3.1.2. Harvesting the Sample

Samples have been take from lateral and medial condyles, and tibial plateau of the OA patient that have been undergone for the TKA surgery. We should state again that these part of bones are routinely removed during the TKA. Patient has been checked for HCV, HBV, AIDS, COVID19 and other routine pre-operative test to eliminate any chance of contamination risk from the study. Inside the operating room taken tissue

samples were cut, diced and smashed to up to 6-7mm of species and then aliquoted in 50ml of sterile falcon tubes. Sufficient transfer media has been added to the specimens inside the falcon to emerge gotten tissue completely. Then falcons have been located in the cool box which had been filled with ice to establish the temperature needed.



Figure 3.1. Diced and smashed samples in transfer medium.

3.1.3. Digesting the Specimens

As soon as we arrived our laboratory, Falcon conical tubes have been removed from cool box, Alcohol sprayed and centrifuged in 1200rpm for 10 minutes and supernatant medium has been discarded. Prepared digesting media solution has added to conical falcons to immerge the remained pallet. 0.5% of prepared collagenase type II solution in W/V ratio have been added and gently mixed. Specimens have been incubated overnight (16h) in 5%CO2 and 37 degree of Celsius temperature at humidifier incubator.

3.1.4. Seeding Cells

Media removed from incubator and strained through 100µm cell strainer to another 50ml sterile falcon tube with the help of bottom tip of the 2ml sterile syringe. Falcon was centrifuged for 5min with 600g in 4°**C**. Pallet washed with prepared washing

medium and previous step repeated to reach the pallet. Supernatant has been discarded and pallet homogenized with small amount of remaining supernatant above to perform cell count. After cell count 20,000 cell/Cm2 has been seeded to one 25Cm2 culture flask (Around 500,000 cells) and flask covered with complete medium. Prepared flask was incubated in 37°C, 5%CO2 humidifier incubator. Growth medium change applied first after 3days then each two days to reach 30%-45% of confluency for subculture.

3.2. Animal Experiment

3.2.1. Material

Ketamine, Xylazine , 20cc syringes, with an 18 gauge x 1.5" needle , 15cc sterile conical tube.

3.2.2. Selecting Animal and Procedure Principles

For our experiment we will use adult Wister Albino strain of rat (200-300gr) which is one of the most available, cost benefit and human strain similar strains. 10 female rats has been provided by saki animal laboratory. Male rats used only to fertilize female rats and leaded to achieving 5 pregnant and 5 non-pregnant female rat which used in the study (Figure10). Reason of selecting this amount of rat is because in the procedure of the our serum production if whole procedure goes successfully our blood drawn to final serum product will be 15cc : 6 cc from 400-450gr rats (144) (since our rat average weight was 250gr, 7-8 cc of total blood has been estimated to be drawn from one rat) and because matter of the fact in our experiment procedure we targeted to accomplish PRMS and NPRS concentration in 10% of total complete media in add-on manner, about 3cc of serum from each rat seen to be sufficient for the experiment alongside with accomplishing the reduction tenet of the animal ethics. (According to other study in FBS-fetal bovine serum-which made to compare different concentration of FBS as growth factor in chondrocyte culture whish shows 2-10% concentration of FBS as growth factor will be satisfactory for the cell growth (145).



Figure 3.2. Wister Albino rats. Left cage is pregnant and Right cage is non pregnant group of female rats.

Process of breeding will be done by natural cross breeding in the cage and pregnancy time would be define as identifying of the vaginal plaque. First day of vaginal plaque identification would be consider as first day of pregnancy.

3.2.3. Producing Pregnant Rat Maternal Serum

I. Anesthesia:

70mg/kg ketamine and 5mg/kg xylazine used as anesthetic drug. Minimum xylazine concentration according to the guidlines has been applied to avoid excess bradycardia as a side effect which may harden the process of cardiac blood sampling. Ketamine has been used as optimal dose of recommended to initiate anesthesia. Prepared concentration has been use intraperitoneally.

II. Blood Drainage method selection:

To include least animal in our experiment we will use cardiopuncture technique for drawing the blood. By the overview done by Kay Stewart and his colleagues at University of Notre Dame (https://www.jove.com/science-education/10246) This

method can gives 40 µl/g rat blood which is up to 10 ml of blood in case of successful procedure from 250gr of rat.

III. Blood sampling (Figure 3.3):

A 20 cc syringe with an 18 gauge x 1.5" needle is selected. Animal has been positioned in dorsal recumbency position. Lateral approach from the animal's left side has been selected for needling. Right against the point of the elbow from the left side measured to be point of needle entrance. The needle is inserted perpendicular to the plane of the table at a point midway on the chest wall as measured dorsoventrally. After the needle being positioned to the measured place, we bevel a needle up a bit, into the chest, and puncture the heart. Slight back pressure applied with the syringe. Until blood has filled the syringe addition aspiration has been delayed. Gentle pressure has been applied to the liver to force additional blood volume into the circulatory system, and ease the withdrawal (Not applied in pregnant rats). Withdrawn blood were placed in 15cm sterile conical tubes.

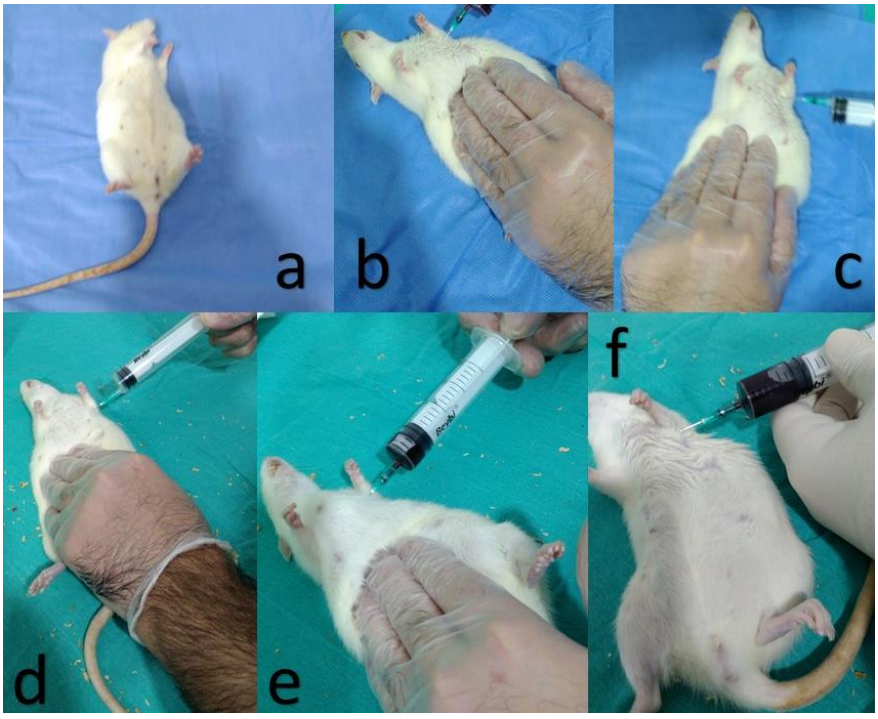


Figure 3.3. Blood sampling. a: Dorsal recumbency position; b,c,d: Site of enterance and lef hand portal system pressure technique; e,f: Blood withdrawel.

IV. Serum extraction (Figure 3.4):

Collected blood centrifuged for 5 min at 1,200 x g and in room temperature to separate the blood into upper and lower layers. Conical tubes were placed in cool box filled with ice, and transfer to laboratory where further placed in 4 degree of Celsius to complete 2hours in purpose of fibrin clot formation. Fibrin clot in the upper layer were squeezed by using sterile curved forceps to separate the serum. Then, fibrin clot were pressed and forced to face downwards near the interface between the two layers. Steps 3 is repeated to maximum serum amount released and fibrin clot interfaced two layers. Furtherer, conical tubes were Centrifuged for 5 min at 1,200 x g and room temperature. Upper layer serum carefully transferred into a 15 ml sterile tube using a sterile pipette in a laminar air-flow cabinet. Collected serums were centrifuged again for 5 min at 1200 x g and in room temprature to remove any remaining red cells. Serum carefully pooled into a new 15 ml sterilized tube using a sterile pipette to avoid contamination by residual red cells at the bottom of the tube. Serum tubes were placed in refrigerator for short time prior to use.

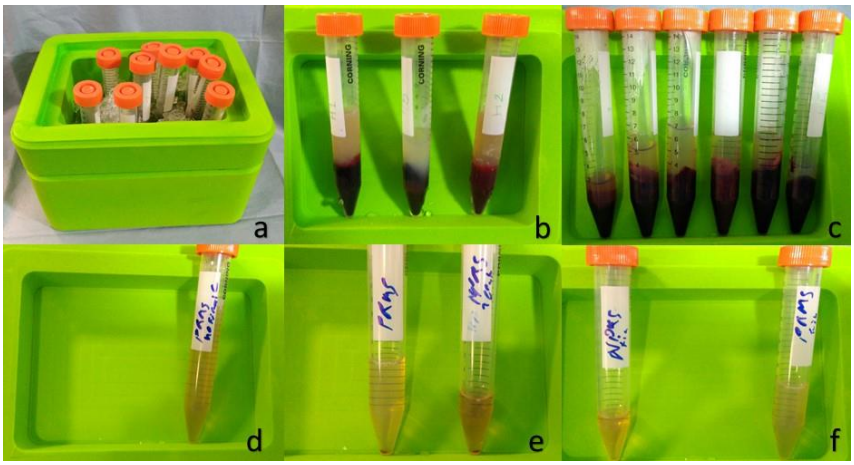


Figure 3.4. Serum Extraction. a:Samples after (1) step transferred to the lab in the cool box; b:Fibrin clot formation after 2h; c:appearence of serum after squeezing fibrin clot and centrifuge tubes; d:pippetted uper serum layer (step 6); e:pelleted remained RBC(step 7); f:Final serum appearance.

3.3. OA-Chondrocyte Secondary Culture:

After reaching the 30%-45% of confluency of the cells, remaining media was removed from culture flask. Then, flask was washed with PBS and trypsinized by 10 minutes of incubation with Trypsin EDTA solution in incubator. Further trypsinization was blocked with prepared washing media solution containing DMEM + 10% FBS. Cells were controlled for detachment level in invert microscope. Cells with media was added to 15ml conical tube and centrifuged for 400xg for 5 minutes to pallet the cells. Supernatant was aspirated without disturbing the pallet. Tip of the conical tube was flicked to loosen the pallet. Cells were further diluted and homogenized by adding the 5ml of culture media to the conical tube. Cells counted and inoculated in 24-well culture plate for 36000 cell/cm^2. Except the main control group, each well treated with related independent variability by 10% of well media in added amount manner. Therefore, negative control group formed as adding NPRS, Positive control group as adding Hanks' Balanced Salts with Ca & Mg, w/o Phenol Red solution, and examination group by adding PRMS. Allocation of groups in 24-well culture plate is shown in figure 10. According to this, All of the A row wells were main control group, B were NPRS group, C were HBSS group and lastly D row labeled as PRMS group. Following of dividing 24-well plate to two 12 well partition for purpose of duplicate the study and increasing the statistics accuracy. Therefore, 1 and 4 column selected for Alician blue stainings, 2 and 5 columns considered as Sirius Red staining, and lastly 3 and 6 columns considered as Alizarin Red staining wells which later is elaborated in staining section below. 24-Well plate was incubated at 5% Co2 in humidifier incubator.

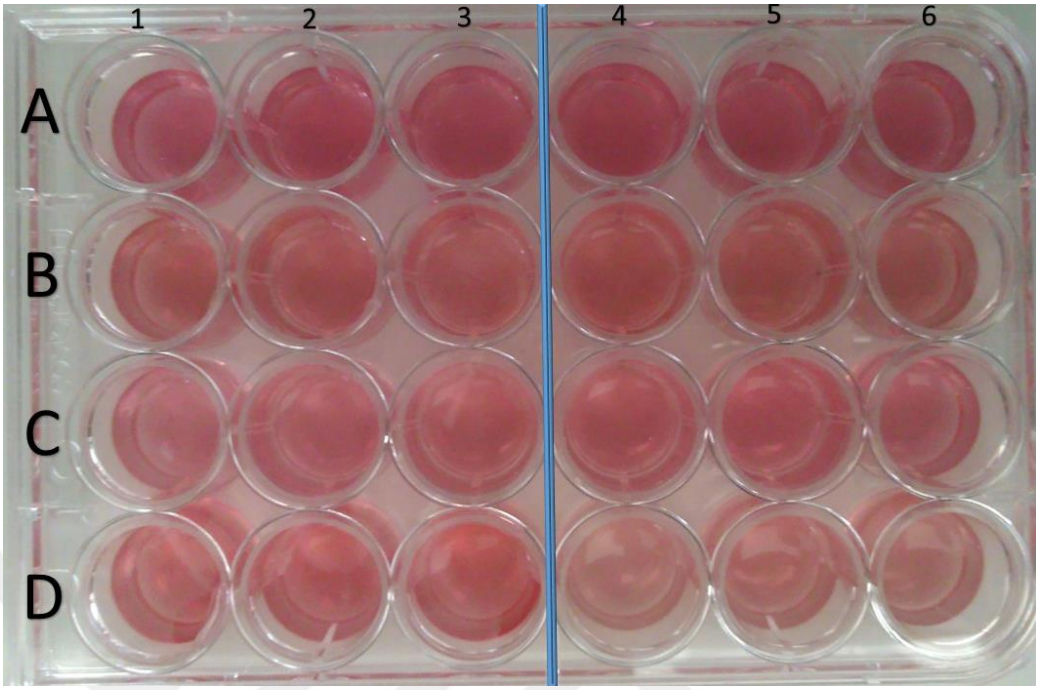


Figure 3.5. Allocation of groups in 24well culture flask.

3.4. Staining:

3.4.1. Alician Blue :

Alician blue solution dye were prepared by adding 0.1M HCL in purpose of targeting strongly sulfated mucosubstances (chondroitin and keratan sulphate) in 1gr : 100ml ratio respectively. First and 4th column of 24well plate were washed with PBS two times. Wells were fixed with 4% of formaldehyde for 20 minutes. After fixation, well were washed with PBS for three times. Then, related wells were stained with Alician Blue prepared solution and incubated for 5hours in room temperature. Wells then were rinsed with 0.1M of HCL. Then washed with PBS for three times. Wells kept in PBS.

3.4.2. Sirius Red :

Sirius red staining method has been inspired from Orriss and his colleagues work (146). Sirius red dye solution were prepared by adding 0.5M acetic acid to reach 50µM

(69µg/ml) concentration solution. Second and fifth columns wells washed with PBS for two times. Related wells were fixed with 70% ethanol for 1h. Wells were washed with PBS for two times and left for air dry. Staining were done by adding the prepared sirius red solution and incubated for 1h in room temperature. Wells were washed with distilled water for two times and kept for air dry.

3.4.3. Alizarin Red :

Alizarin Red solution were prepared by adding distilled water to reach 1% (W/V) solution. Third and Sixth columns of 24-well plate were washed with PBS for two times. Wells were fixed with 10% formaldehyde for five minutes. Then cells were washed with 70% ethanol for three times and kept for air dry. Then, related wells were stained with Alizarin Red and incubated for 5minutes in room temperature. Wells were washed with 50% of ethanol for three times and kept for air dry.

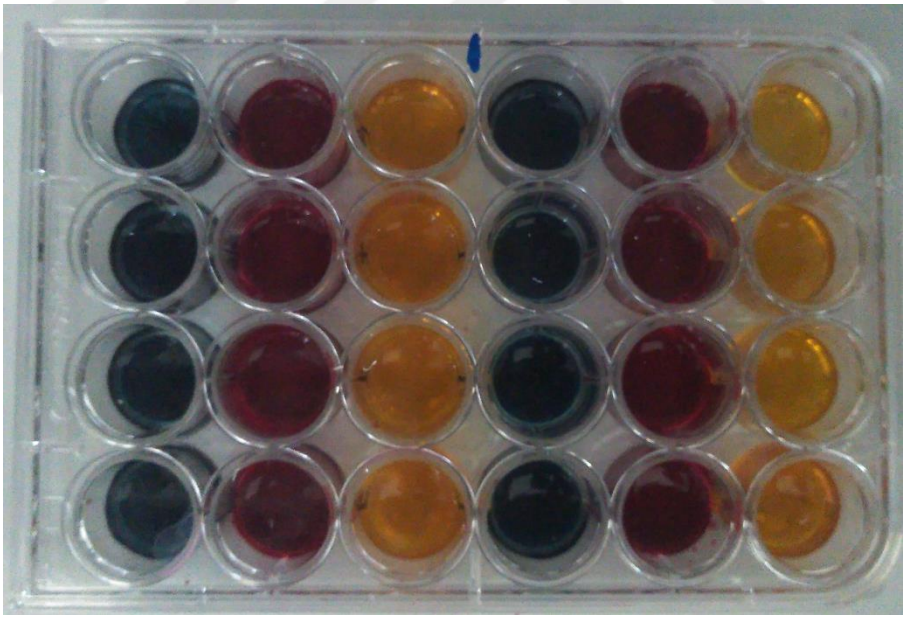


Figure 3.6. 24-Well plate after adding dye solution.

3.5.Analyzing

1) Microscopy: Well plates were analyzed for evaluation of cellular geometric morphology, staining, filopodia, Intercellular interactions, Clustering formation and lipid droplets.

    a) Qualitative analysis : Comparison between groups were done by comparison factors mentioned above.

    b) Quantitative Analysis : Chondron process (filopodia) length, Chondron Count and sirius red staining color value level (Calculated by chondron mean histogram color value) is evaluated by NIH Image J software. Note that lower value refers to higher intensity of the color.

2) An high resolution scan (9600 dpi, 8160 x 11231 pixel) were done from 24 well plate after staining to evaluating the gross macroscopy.

4.RESULTS

4.1. Qualitative:

4.1.1. Chondron Count

With a rapid look at groups under the 10x magnification a huge increase and different between PRMS group and other groups is Irrefutable. Even in higher magnifications this difference can be seen. There is a bit higher Chondron Count is also distinguishable between NPRS group and main control group without any extra media. The huge difference in cell quantity between HBSS group is also clearly understandable. Accordingly we can state that the cell and chondron quantity shown to have the huge increase by adding the PRMS into the cultured cells. Additionally, a mild increase in NPRS increase is not looking so meaningful because of no huge difference. Quantitative statistical analysis confirmed this thought as well (Figure 4.1.).

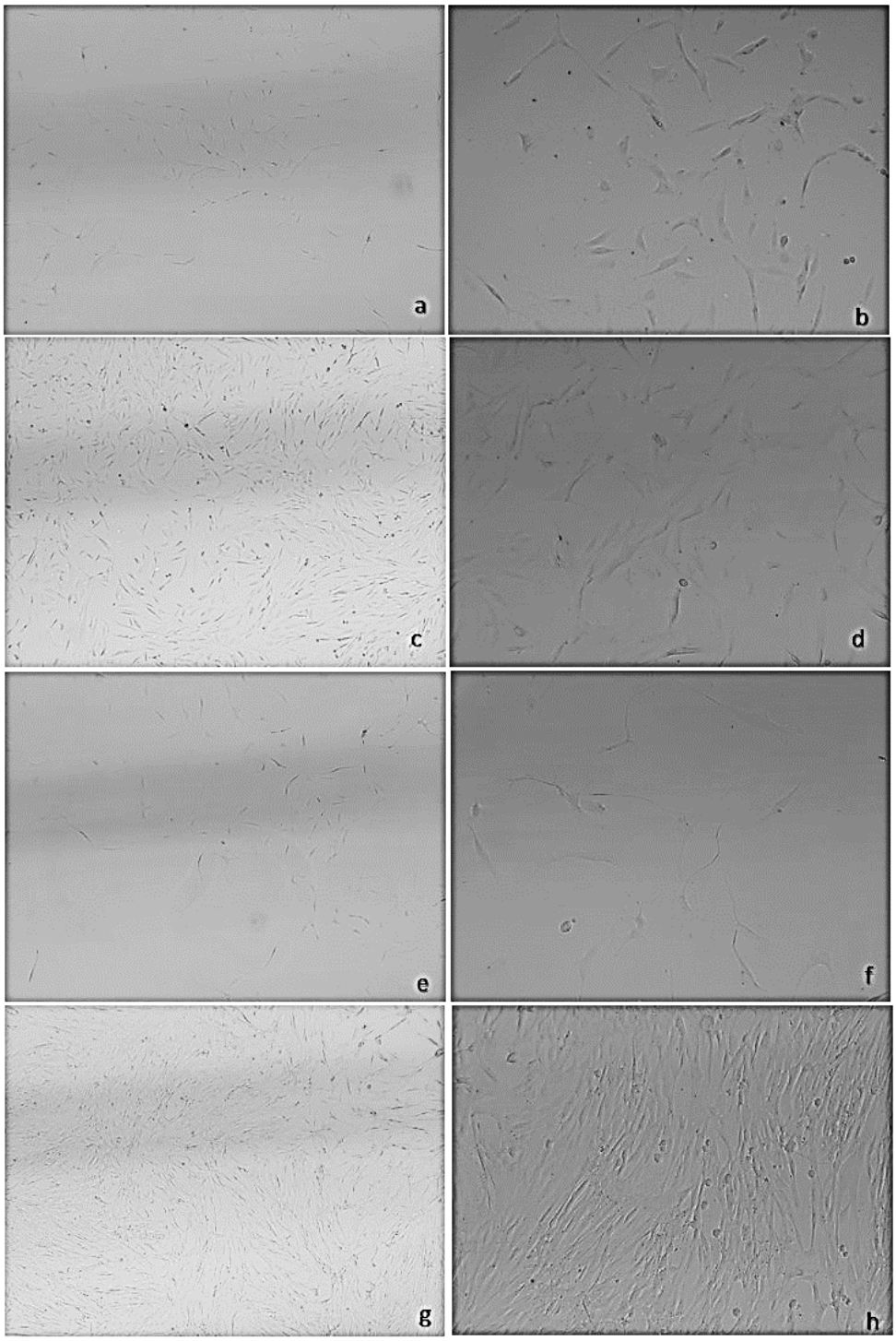


Figure 4.1. Cell line microscopy after 10days from secondary culture. Prior to staining. Left column is X4 objective lens and right column is X10 object lens magnificaiton. a,b:Main control group; c,d:NPRS group; e,f:HBSS; g,h:PRMS group.

4.1.2. Cell Geometry :

PRMS groups shows much more better polygonality and round shaped geometry of chondron which related to normal chondrocytes behavior. This may be shown the ability of PRMS not to only increase the growth activity of the chondrocyte but also its potential in reverse the mal-phenotypic behavior of the OA-Chondrocyte toward the normal activity which can promising its crucial role for treating OA also invivo. Also the bizarre shapes of chondron and high shape variability among of elongation in shape in other groups specially in HBSS group as our positive control group confirm the abnormal activity of OA cells in all the groups except PRMS groups (Figure 4.2) (also note Figure 4.7).

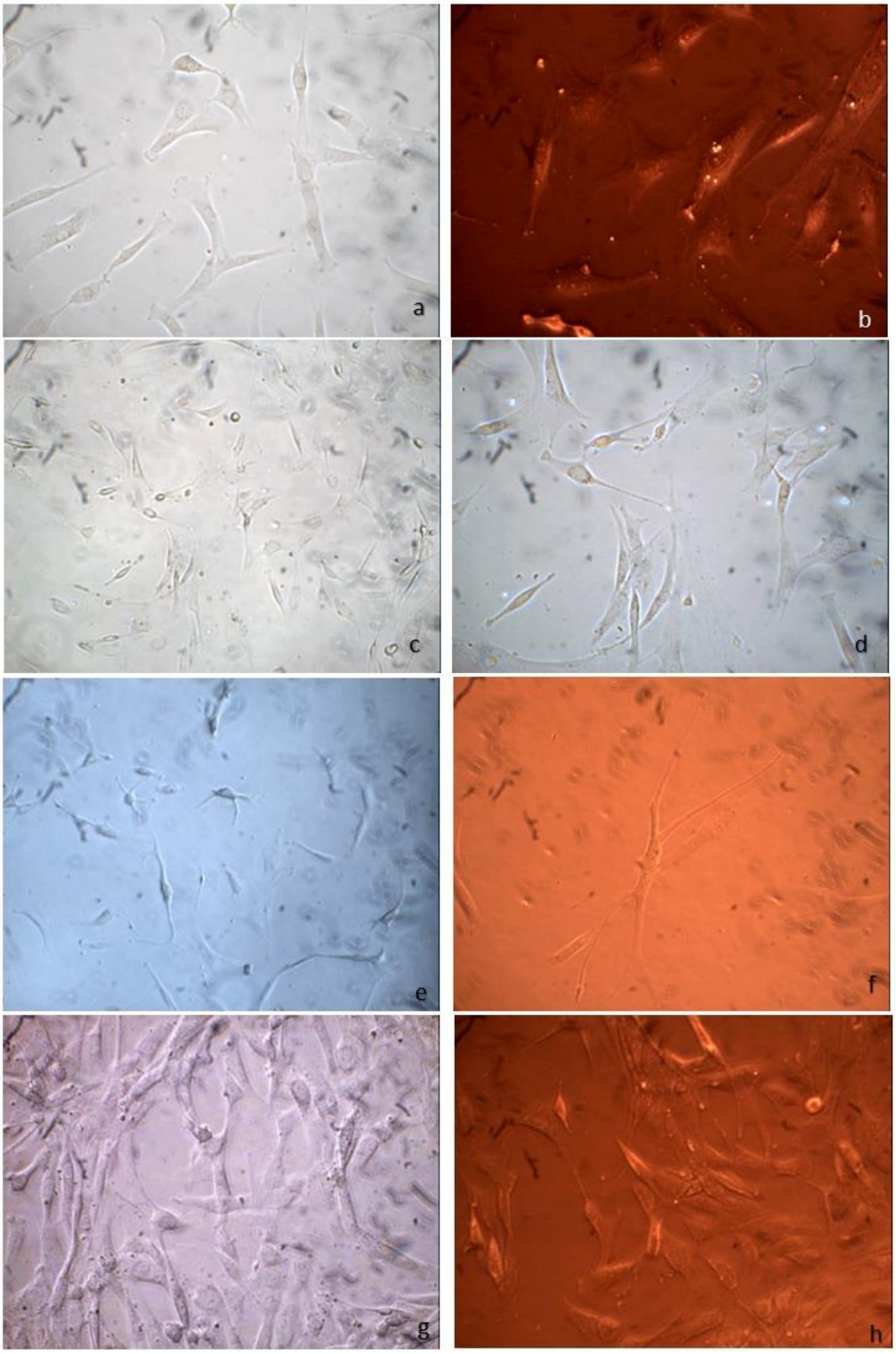


Figure 4.2. Morphologic comparison. Left column with microscope 20X objective and right column with X40 objective lens. a,b:Main control group; c,d:NPRS group; e,f:HBSS; g,h:PRMS group.

4.1.3. Chondron Processes (Filopodia):

By a general observation at the first look of the microscopy, the length of the chondron processes in other groups than PRMS groups catches the eyes. It's quite clear that in other groups than PRMS group we have more length of processes of chondrons which resembles fibroblast morphology. Also, as we mentioned in introduction part it can resemble mobile chondrocyte morphology which also a characteristics of the abnormal behavior of the chondrocytes. In addition, we can see more multi-polar processes in HBSS, PRMS and main control group than PRMS group (Figure 4.3 and 4.7).

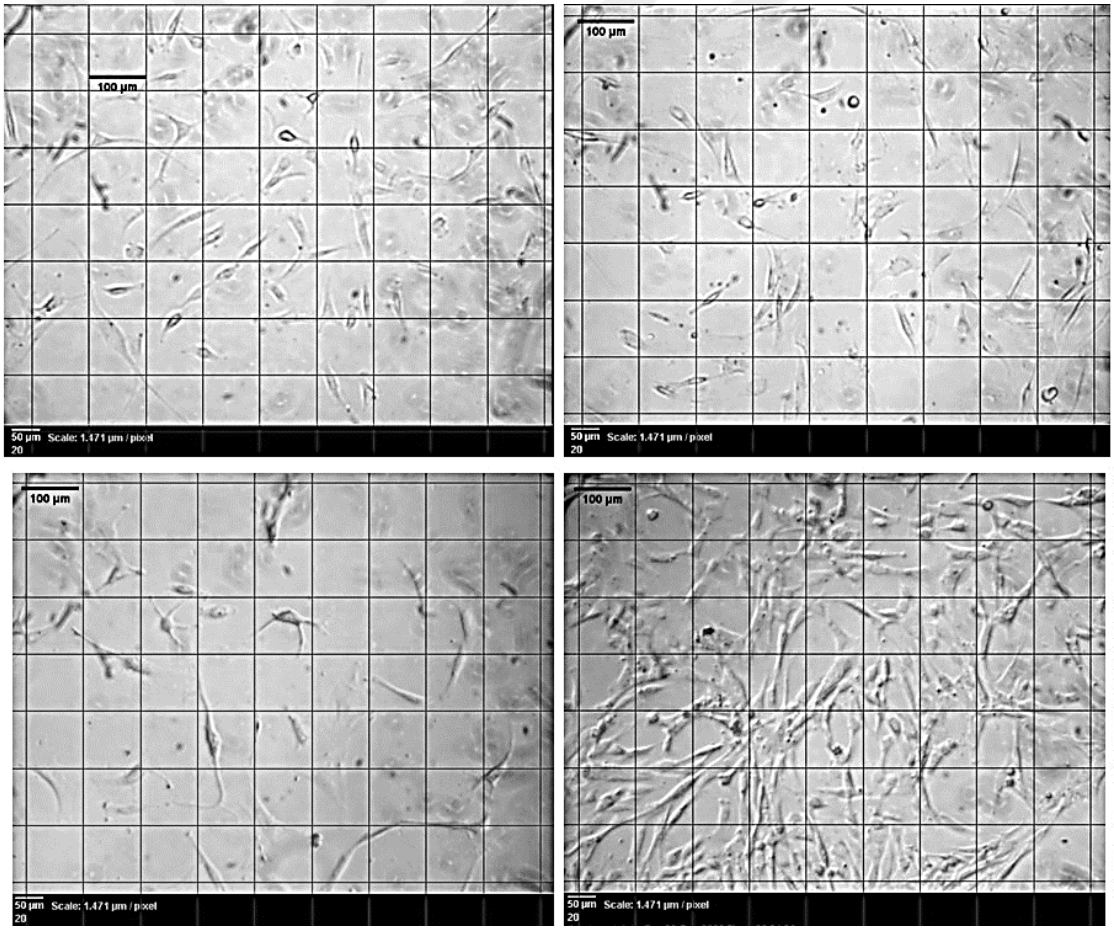


Figure 4.3. Chondron Length. Upperleft: Main control group; Upper right: NPRS; Lower left: HBSS; Lower Right: PRMS.

4.1.4.Presence of the Lipid Droplets :

As mentioned in introduction part lipid droplets can be a good indicator for metabolic activity of the chondrocyte. By little precise inspection of the chondrons between the groups, lipid droplets seems to be much more in PRMS group, but its also least in HBSS group. The reason for this can be explained as no high metabolic activity in HBSS group because of the acidity made by HBSS which least cell and chondron count also confirm this idea. However, we should declare that there is no huge difference was seen between PRMS, NPRS and Main control group was seen. Maybe lipid droplet count comparison should be better done with electron microscopy techniques to have a detailed comparison toward the count, size and precise identification of the droplets (Figure 4.4 and 4.7).

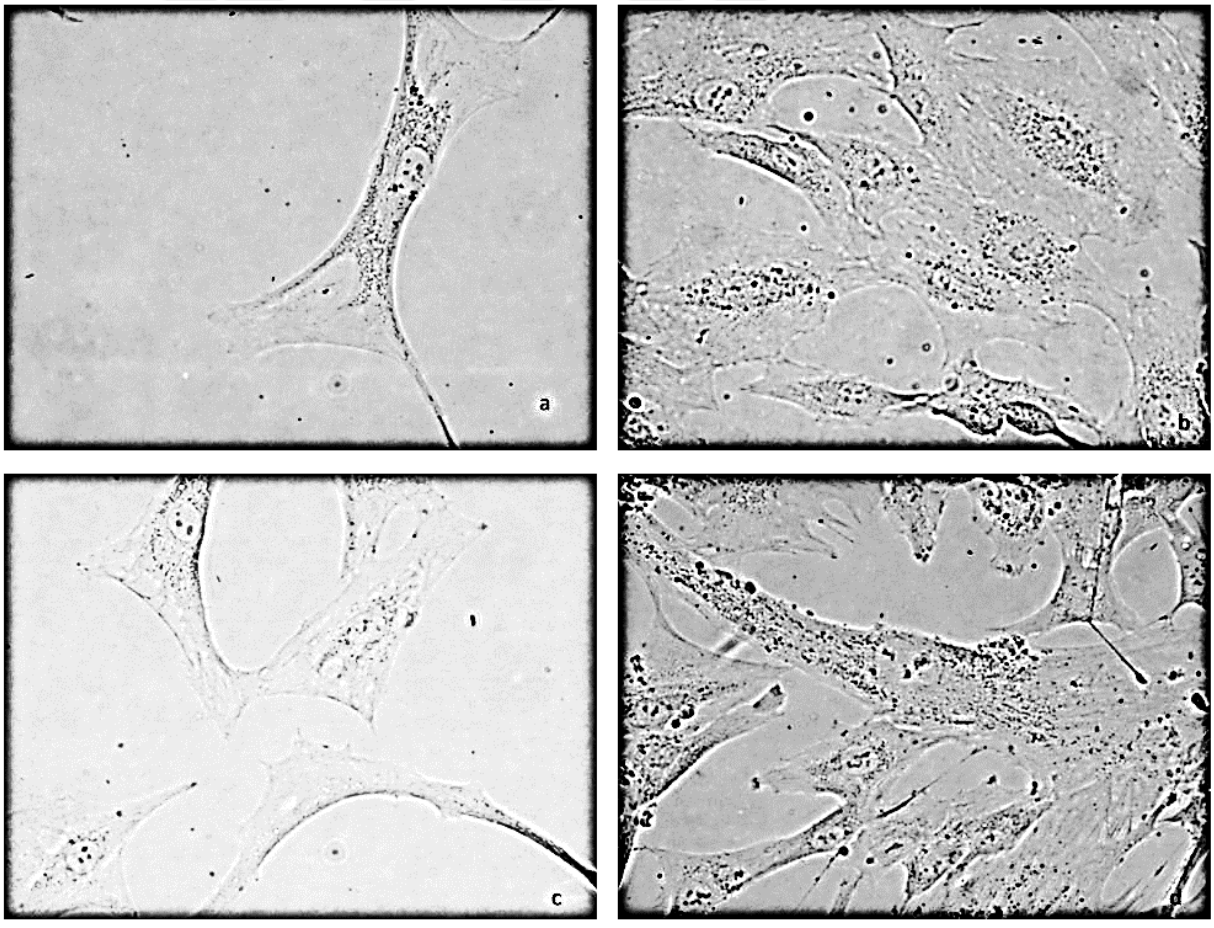


Figure 4.4. Lipid droplets. a:Main control group; b:NPRS; c:HBSS; d:PRMS.

4.1.5. Intracytoplasmic Bold Dark Spots :

Usually dark spots in cytoplasm specially near the nucleus resembles Golgi apparatus, Endoplasmic reticulum and protein expression related area of the cell in general. Here also Intracytoplasmic bold-dark spots despite the fact that we can not distinguish their identity, but it may be an indirect estimation of cell protein expression activity. Accordingly, we can say we have higher protein expression activity of the chondrocytes in PRMS groups which surely partly due to of higher matrix protein level production of the chondrocytes in this groups(This is also confirmed after staining process) (Figure 4.4 and 4.7).

4.1.6. Chondroptosis :

HBSS group is a beautiful sample that we can find the chondron without chondrocytes areas in our studies. These findings also found in main control group and NPRS group but not in PRMS group.

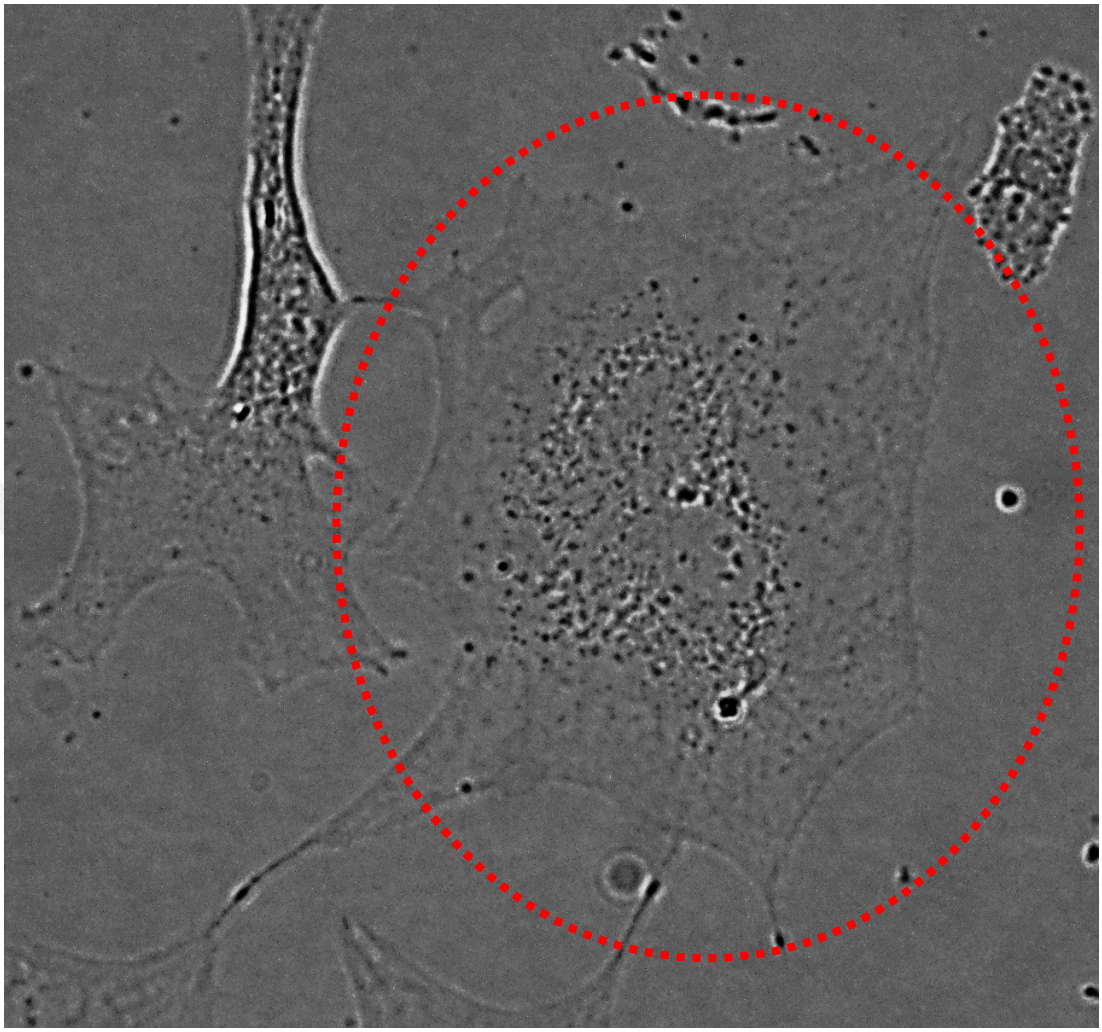


Figure 4.5. Chondroptosis.

4.1.7. Clustering:

We could not found any dominant clustering or any isogenous chondrocyte formation in any of the groups. The reason can be because of the fact that clusters formation can be happen after some other furtherer passages in cell line or It may not be a good factor for the comparison between groups in OA invitro studies atall.

4.1.8.Staining :

After staining process, stained wells were analysed macroscopically by scanning 24well plate (9600 dpi, 8160 x 11231 pixel) and microscopically.

a) Microscopy: In the microscopic inspections of the wells, with Alician blue staining intensity and density of the Alician blue was higher in PRMS group which shows the higher amount of the strongly sulfated mucosubstances (chondroitin and keratan sulphate). As its expected Alician blue dye stained intraterritorial matrixes where the chondroitin and keratan sulphate expected to present. By staining with sirius red dye, the higher amount of collagen in PRMS group was observed obiously and that was not only because of higher amount of the cells but also the red color intensity of the extracellular matrix seems to be much more intensive than other groups. These findings reflecting the higher amount of the collagen in PRMS group clearly. With Alizarin Red staining we didn't find any stained area in all the groups which shows no sign of ossification process or calcium depletion in any of groups (figure 4.6, 4.7, 4.8).

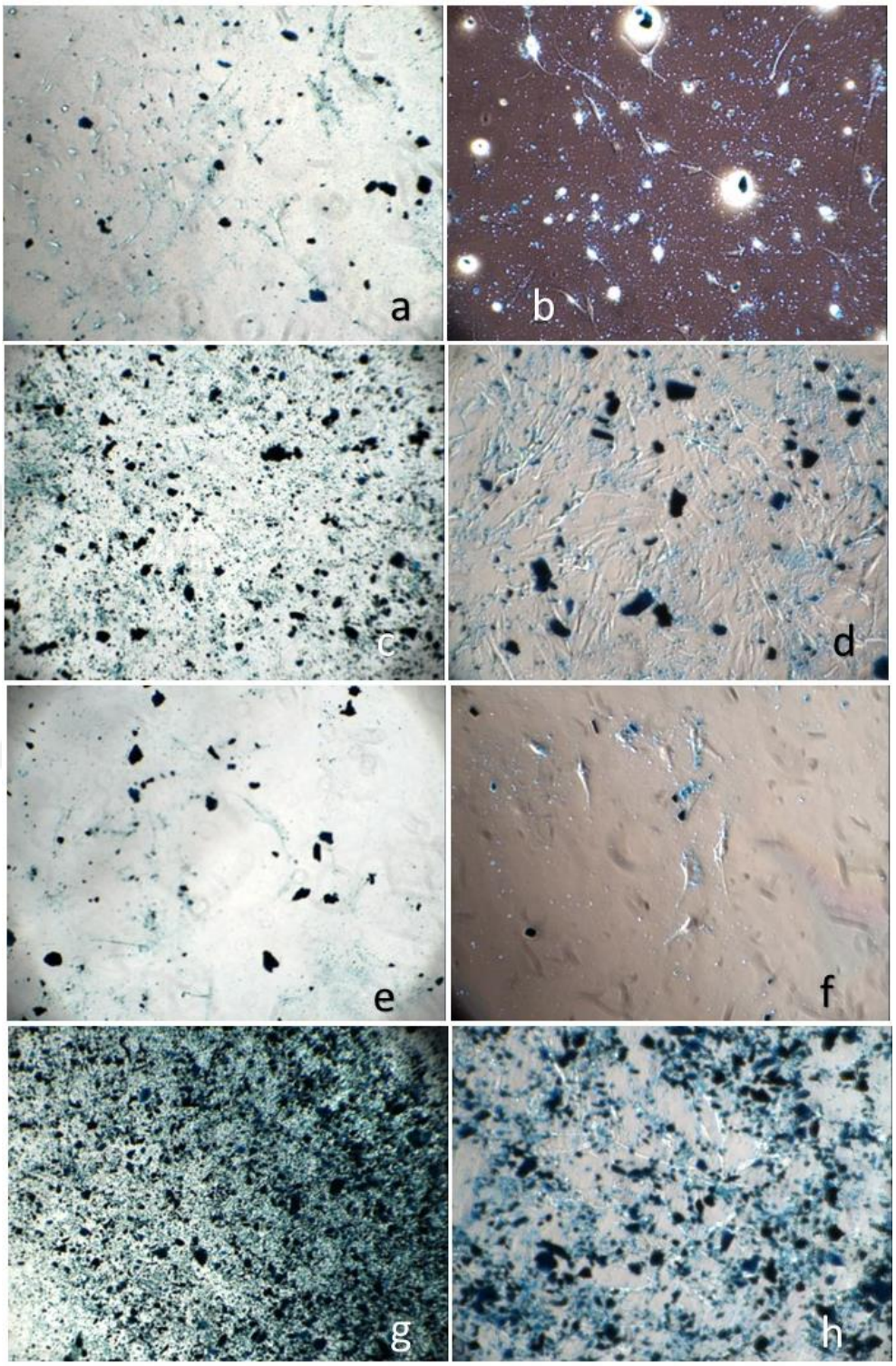


Figure 4.6. Alician Blue staining. Left column X10 objective lens-Bright field, Right column X20 objective lens-Phase contrast. a,b:Main control group; c,d:NPRS; e,f:HBSS; g,h:PRMS.

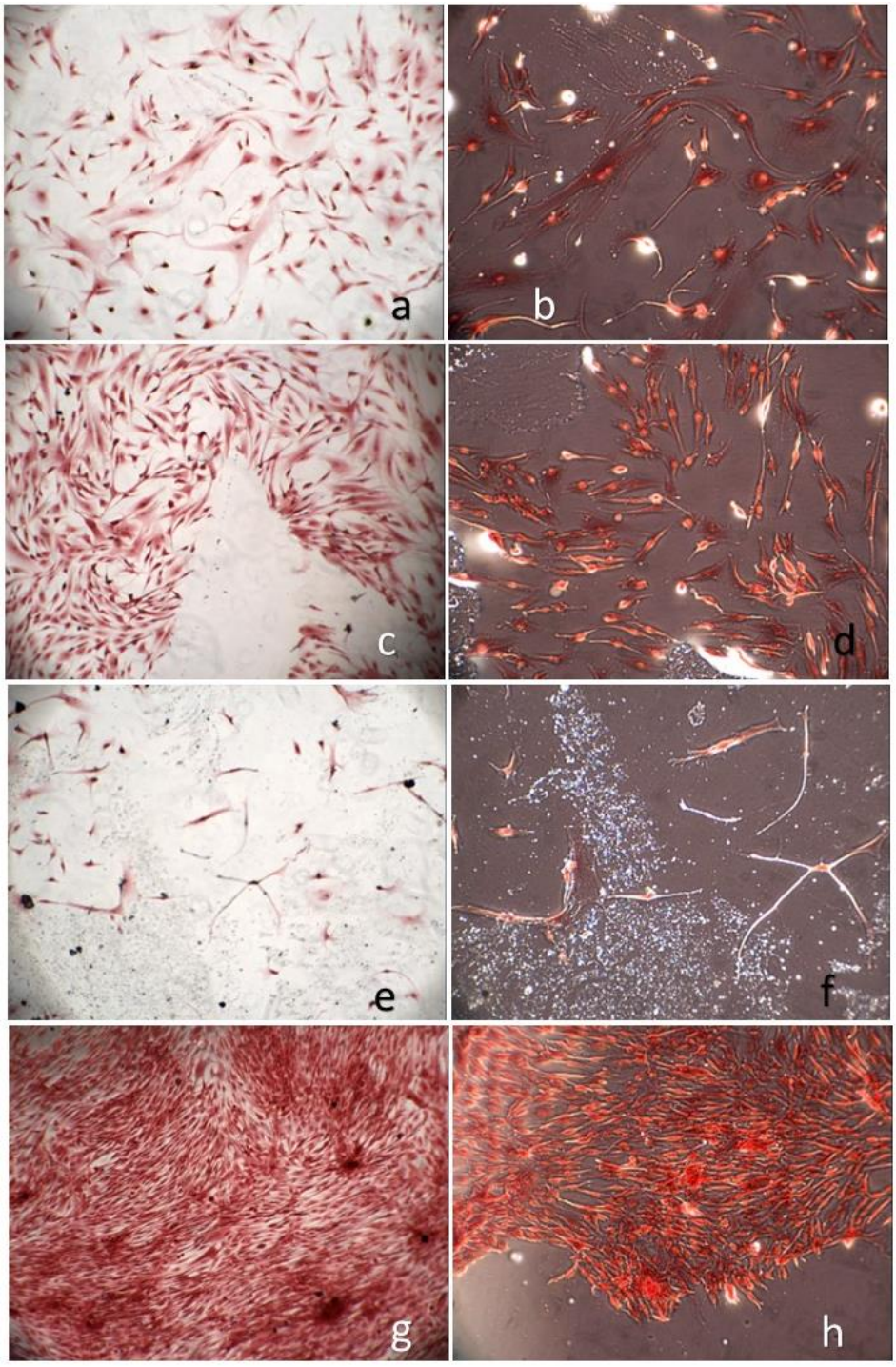


Figure 4.7. Sirius Red staining. . Left column X10 objective lens-Bright field, Right column X20 objective lens-Phase contrast. a,b:Main control group; c,d:NPRS; e,f:HBSS; g,h:PRMS.

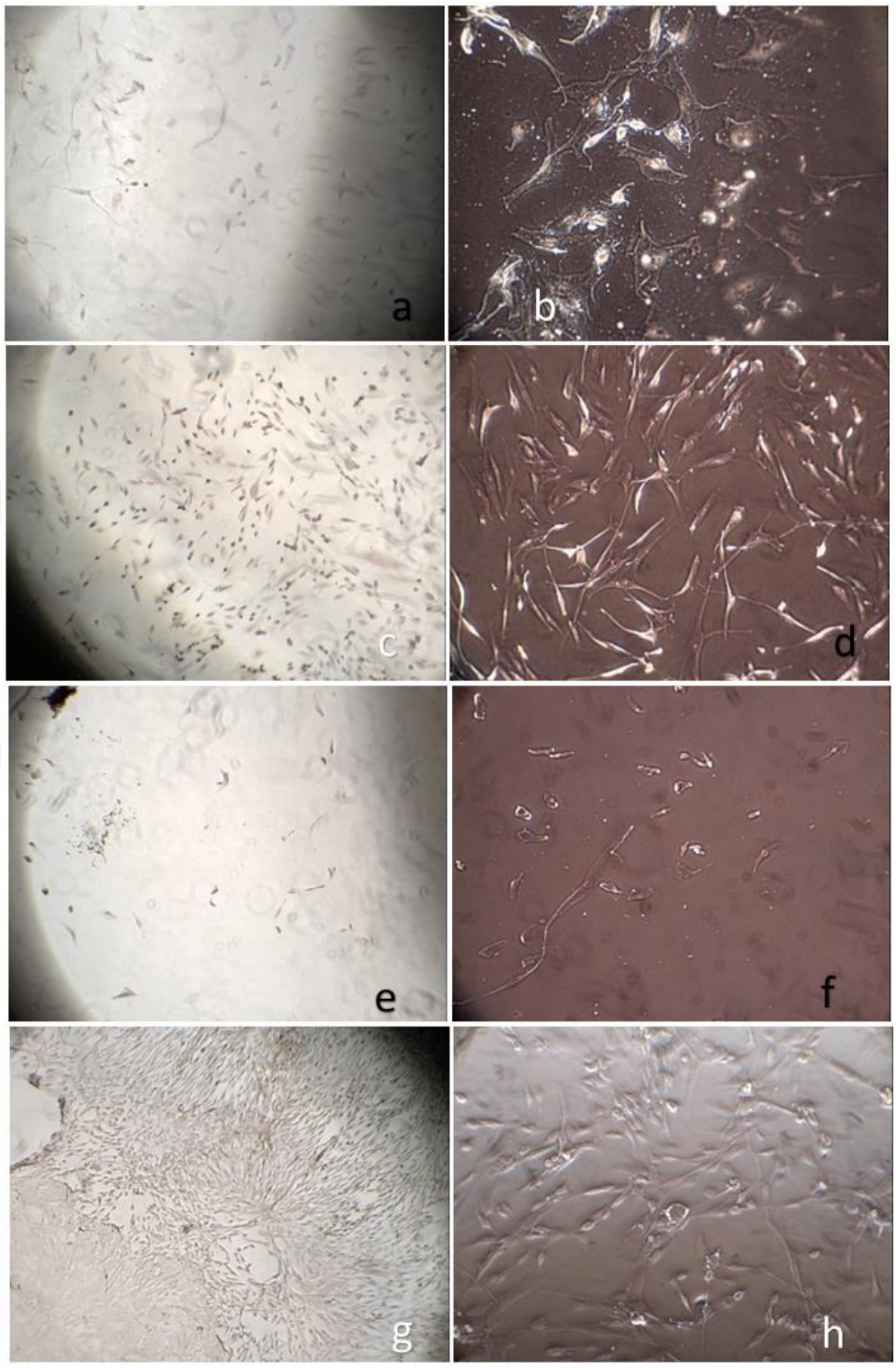


Figure 4.8. Alizarin Red staining. Left column X10 objective lens-Bright field, Right column X20 objective lens-Phase contrast. a,b:Main control group; c,d:NPRS; e,f:HBSS; g,h:PRMS.

b) Macroscopy: After scanning to achieve high resolution (9600 dpi, 8160 x 11231 pixel) image, by zooming in to groups, our finding in microscopic scale was also confirmed here as macroscopic scale as well. Alician blue and sirius red staining were cover highest surface area of the wells related for PRMS group and least for HBSS group (Figure 4.9,4.10).

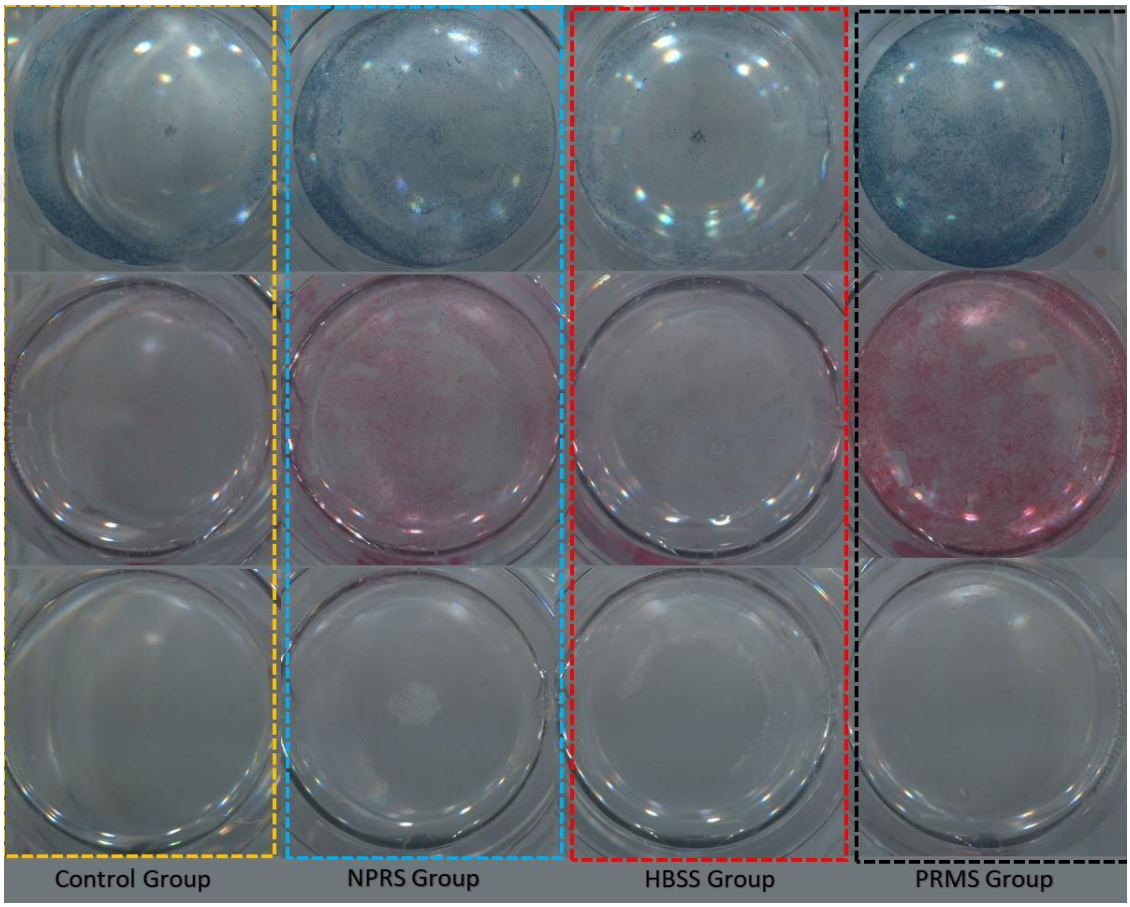


Figure 4.9. Magnified image of gross macroscopy. First row: Alician Blue; Second row:Sirius Red; Third row: Alizarin Red.

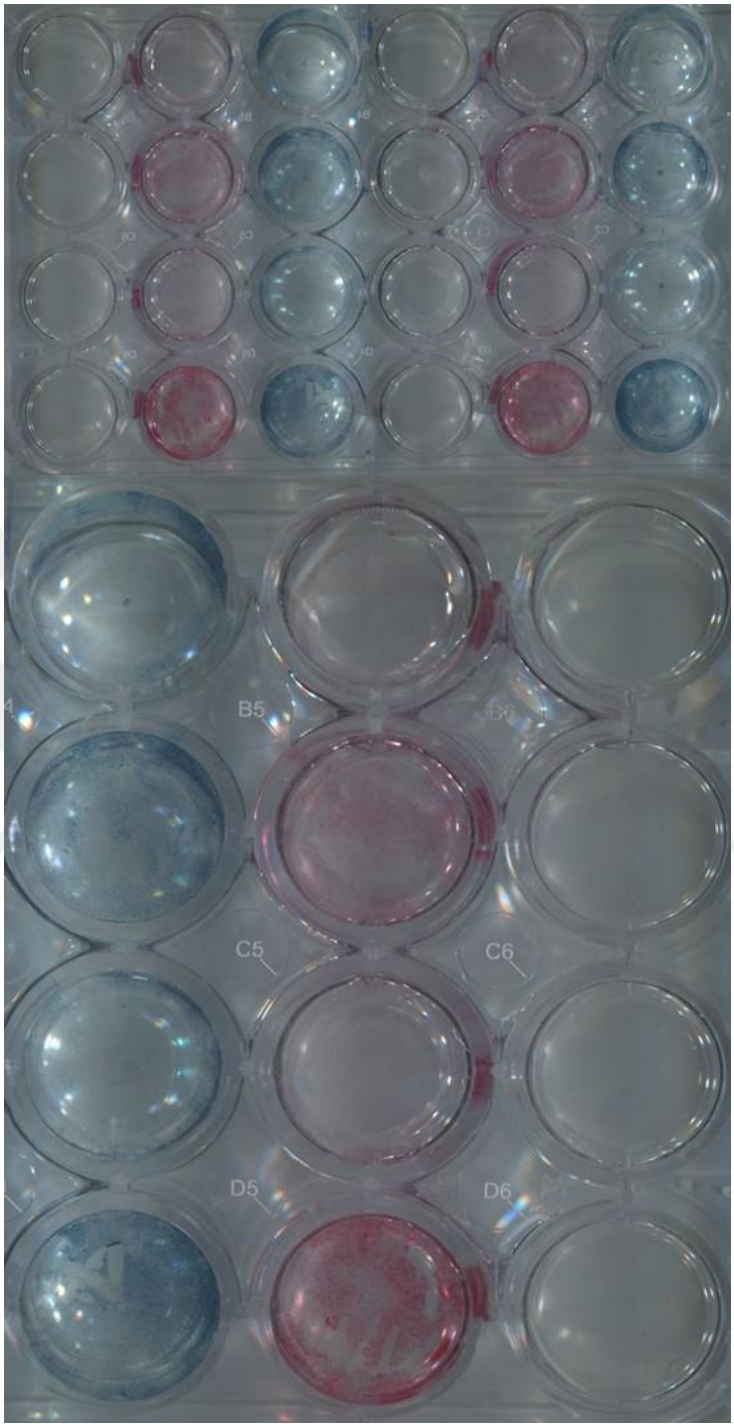


Figure 4.10. Gross macroscopy view. Top: from left 1st and 4th columns are Alizarin Red staining, Second and 5th columns were stained by Sirius Red, third and 6th columns were stained with Alician Blue; Bottom: first column is Alician Blue, Second is Sirius Red, Third column is Alizarin Red. Note that in both pictures first rows related to main control, Second row is NPRS, Third row is HBSS and 4th row is related to PRMS.

4.2.Quantitative

Chondron processes lengths, Sirius Red staining red color histogram value (figure 4.11), and chondron counts were quantitively measured with NIH Image J software and datas were analysed by One-way Anova method for statistics.

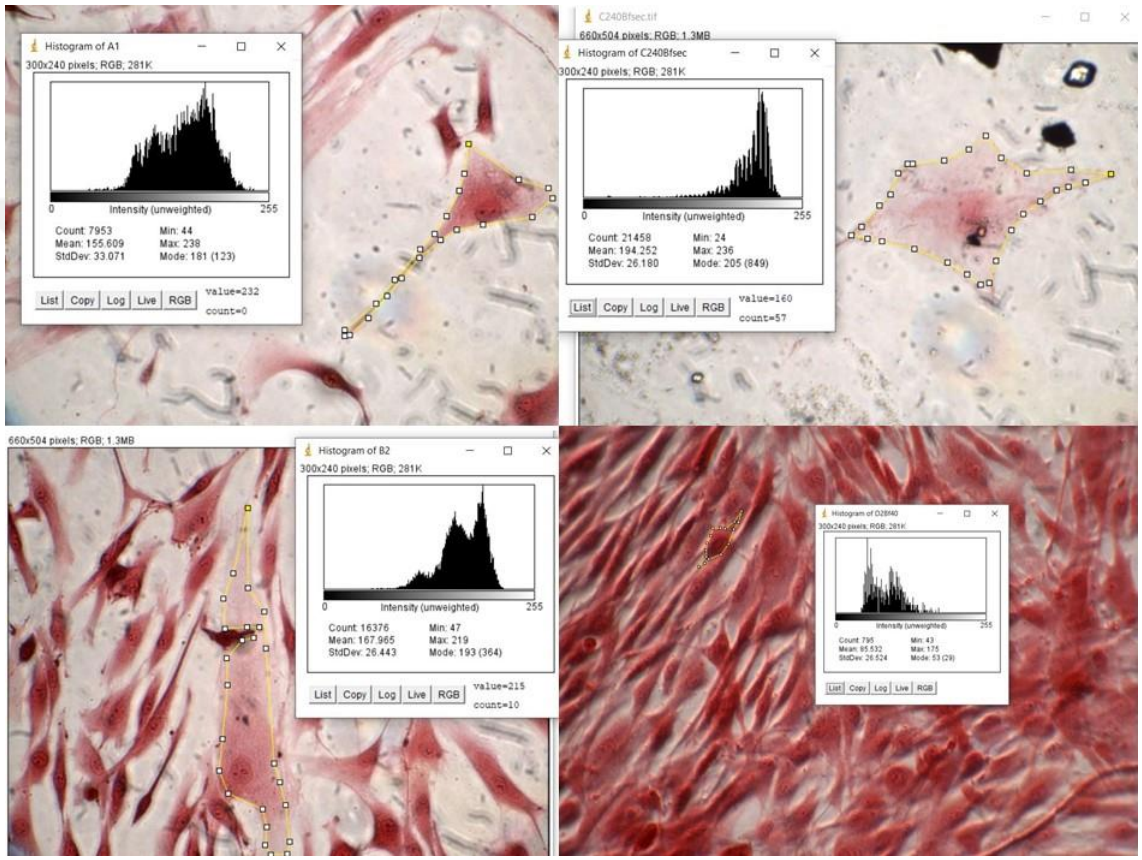


Figure 4.11. Color evaluation histogram process. Upperleft: Control group; Upperright:HBSS group; Lowerleft:NPRS; lowerright:PRMS group.

4.3.Statistics

4.3.1 Descriptive Statistics:

Chondrocyte Length:

Separately for each group in the chondron length study, for the first control group (Control without adding anything extra) in the study of 36 observed cells, the maximum observed chondron length was 219 µm and the minimum was 3 µm. (Range = 216 µm) The mean cell size was 75.5 (±51.869) µm, which 37 µm was the length of the most frequent cells in this group (8.5%).

These statistics for the second group (NPRS) were measured for 132 cells, the average measured length of these cells was 33.64 (± 21.877) µm, with the lowest 1 and highest length of 100 µm.

These calculations were also performed for the third group (HBSS). The mean length of cells in this group was 110.30 (± 48.463) µm, with a maximum length of 190 µm and a minimum of 6, which 119 µm was the highest frequency of cells(20%).

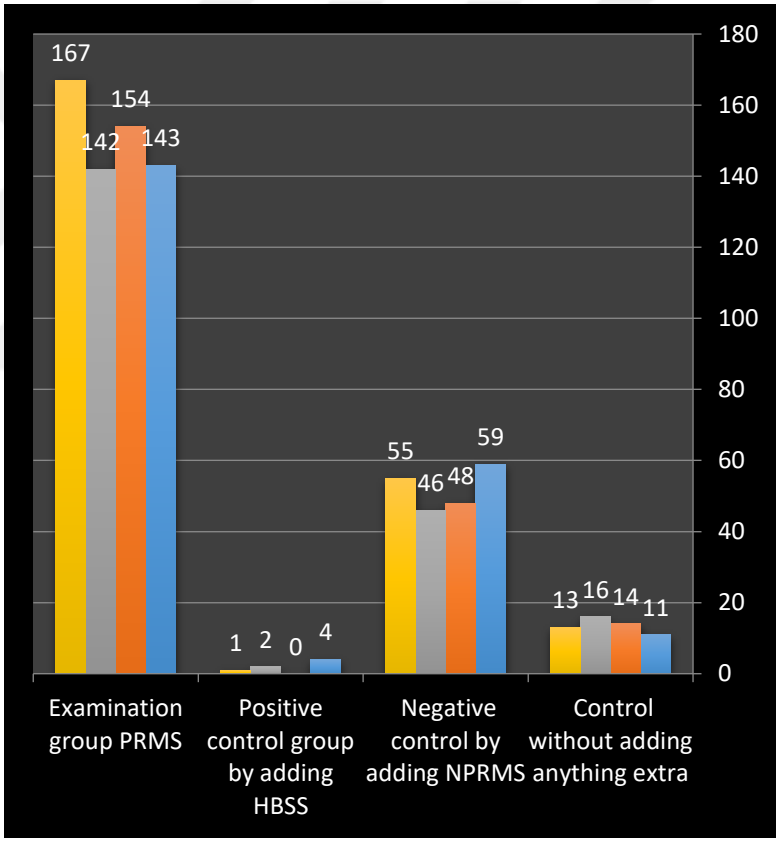
For the experimental group, the mean length of 100 cells was 20.26 (±13.484) µm with a maximum of 74 and a minimum of 1 µm, which the most repetitive length measures was 18 µm.

Table 4.1. Descriptive statistics of chondrocytes length.

Main control without any Extra added material	N	Valid	36
		Missing	0
	Mean		75.50
	Median		68.00
	Mode		37
	Std. Deviation		51.869
	Variance		2690.429
	Range		216
	Minimum		3
	Maximum		219
Negative control by adding NPRS	N	Valid	132
		Missing	0
	Mean		33.64
	Median		31.50
	Mode		13
	Std. Deviation		21.877
	Variance		478.600
	Range		99
	Minimum		1
	Maximum		100
Positive control group by adding HBSS	N	Valid	10
		Missing	0
	Mean		110.30
	Median		115.50
	Mode		119
	Std. Deviation		48.463
	Variance		2348.678
	Range		184
	Minimum		6
	Maximum		190
Examination group PRMS	N	Valid	100
		Missing	0
	Mean		20.26
	Median		18.50
	Mode		18
	Std. Deviation		13.484
	Variance		181.811
	Range		73
	Minimum		1
	Maximum		74

Chondrocyte Count:

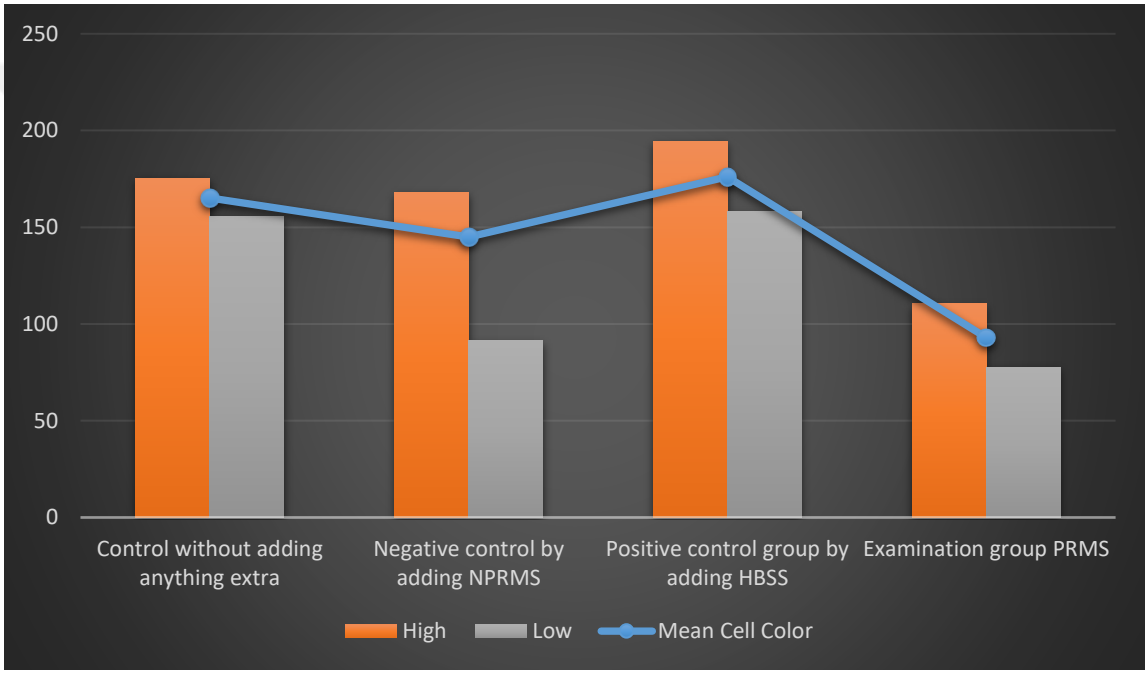
Chondrocyte counts in each group that were studied with 4 different plates averaged 13.5 (±2.08) in the control group, which in the other two control groups, NPRS and HPSS, were 52 (±6.05) and 1.75(±1.7) respectively. On the other hand, in the experimental group (PRMS), the average number of cells observed in the 4 studied plates reached 151.5 (±11.67), which is analyzed in the next section.



Graph 4.1. Detailed data of chondrocyte counting in each plate.

Chondrocyte Color:

The color of chondrocytes was calculated as a number that in the first control group the average color intensity of chondrocytes was measured 165.18 (± 8.07) and in the next two control groups NPRS and HBSS it was 144.95 (± 36.30) and 176.06 (± 20.99), respectively. But in the experimental group, the mean was 93 (±14.46). Note that lower value refers to higher intensity of the color.



Graph 4.2. Color range and mean of chondrocytes in each plate.

Table 4.2. Descriptive statistics of chondrocytes count and color.

Cell Plate		Plates	Range	Minimum	Maximum	Mean	Std. Deviation	Variance
Main control	Chondron Count	4	5	11	16	13.50	2.082	4.333
	Chondron Color	4	19.761	155.609	175.370	165.18767	8.078679	65.265
Negative control by adding NPRS	Chondron Count	4	13	46	59	52.00	6.055	36.667
	Chondron Color	4	76.651	91.314	167.965	144.95175	36.302080	1317.841
Positive control group by adding HBSS	Chondron Count	4	4	0	4	1.75	1.708	2.917
	Chondron Color	4	36.367	157.885	194.252	176.06850	20.996497	440.853
Examination group PRMS	Chondron Count	4	25	142	167	151.50	11.676	136.333
	Chondron Color	4	32.852	77.558	110.410	93.00075	14.463949	209.206

4.3.2 Analytical Statistics:

Evaluation the recorded data in terms of distribution and whether they follow the normal distribution in SPSSv21 software using the One-Sample Kolmogorov-Smirnov Test, we proved that the data follow the normal distribution. According to Table 4-3, which shows the results of the One-Sample Kolmogorov-Smirnov Test, the significance level of this test for the three variables of color, number and length of chondrocytes is calculated to be 0.158, 0.332 and 0.729, respectively, which is the normal distribution of data. And proves the ability to use parametric tests such as One-Way ANOVA .

Table 4.3. One-Sample Kolmogorov-Smirnov test.

		Chondron Color	Chondron Count	Chondron length
N		16	16	16
Normal Parameters[a,b]	Mean	144.80217	54.69	61.06
	Std. Deviation	38.648622	61.131	50.908
Most Extreme Differences	Absolute	.282	.237	.172
	Positive	.135	.237	.172
	Negative	-.282	-.186	-.140
Kolmogorov-Smirnov Z		1.127	.946	.689
Asymp. Sig. (2-tailed)		.158	.332	.729

a. Test distribution is Normal.

b. Calculated from data.

One-Way ANOVA Test was used to prove the relationship between different groups in the study of variables. In this analysis, a significant difference was observed between the groups in terms of all three variables. Table 4-4 shows the P-Value of each of the variables.

Table 4.4. One Way ANOVA test.

		Sum of Squares	df	Mean Square	F	Sig.
Chondron Color	Between Groups	16306.245	3	5435.415	10.694	**.001**
	Within Groups	6099.494	12	508.291		
	Total	22405.740	15			
Chondron Count	Between Groups	55514.688	3	18504.896	410.650	**.000**
	Within Groups	540.750	12	45.063		
	Total	56055.438	15			
Chondron length	Between Groups	22172.188	3	7390.729	5.310	**.015**
	Within Groups	16702.750	12	1391.896		
	Total	38874.938	15			

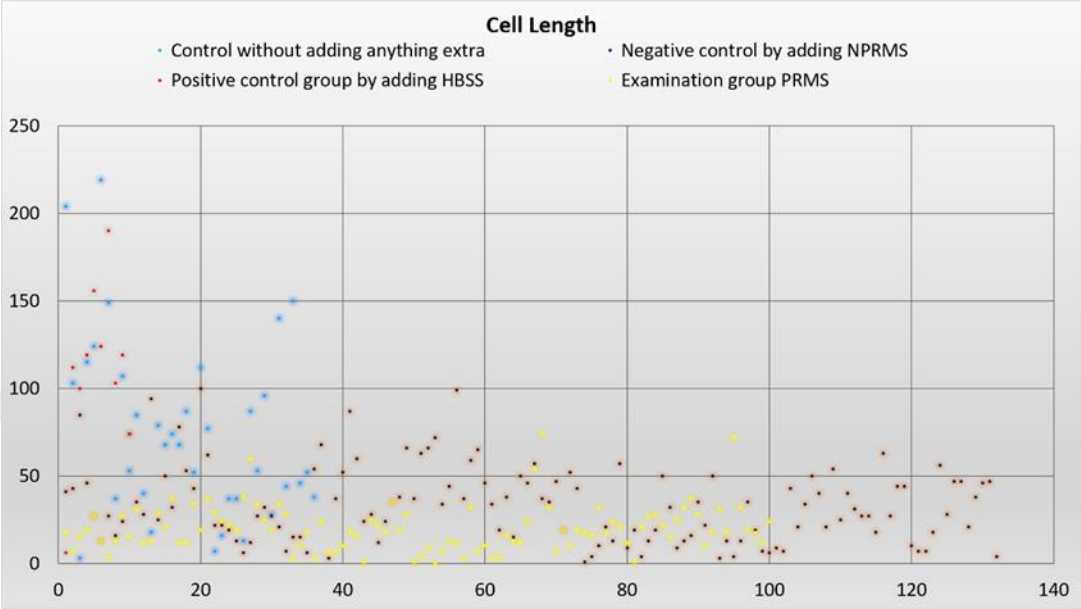
In order to further investigate the significant relationship that can be seen between the groups, the Scheffe table shown in Table 4-4 was used. In this table, compared to the color of chondrocytes in comparison with the experimental group and the other three groups, the difference of the means is negative, which shows the significant difference between these groups in this case with a significance level of 0.006, 0.048 and 0.002, respectively for Control group, NPRS and HBSS.

In the study of the number of chondrocytes, a significant level of difference between the means of the three groups and the experimental group of all three groups was reported to be less than 0.001, which indicates a significant difference in the number of chondrocytes counted.

Comparing the length of chondrocytes (Graph 4-3), only a significant level less than 0.05 was calculated between the experimental group and the third control group (HBSS) (P = 0.028).However, there was no significant difference between the other two control groups and the experimental group, but the difference between the means in the table is considerable.

Table 4.5. Scheffe (Multiple comparisons).

Dependent Variable	(I) Cell Plate	(J) Cell Plate	Mean Difference (I-J)	Std. Error	**Sig.**	95% Confidence Interval	
						Lower Bound	Upper Bound
Chondron Color	Examination group PRMS	Control without adding anything extra	-72.186917*	15.941945	**.006**	-123.77305	-20.60078
		Negative control by adding NPRS	-51.951000*	15.941945	**.048**	-103.53713	-.36487
		Positive control group by adding HBSS	-83.067750*	15.941945	**.002**	-134.65388	-31.48162
Chondron Count	Examination group PRMS	Control without adding anything extra	138.000*	4.747	**.000**	122.64	153.36
		Negative control by adding NPRS	99.500*	4.747	**.000**	84.14	114.86
		Positive control group by adding HBSS	149.750*	4.747	**.000**	134.39	165.11
Chondron length	Examination group PRMS	Control without adding anything extra	**-55.250**	26.381	**.274**	-140.62	30.12
		Negative control by adding NPRS	**-13.250**	26.381	**.967**	-98.62	72.12
		Positive control group by adding HBSS	-94.750*	26.381	**.028**	-180.12	-9.38



Graph 4.3. Point distribution of chondron length in 4 groups.

5.DISCUSSION

According to our hypothesis, we approved the high chondrogenesis induction ability of the pregnant rat maternal serum on OA-Human chondrocyte cell line invitro.

Morphologic behavior, chondron length, amount of collagen and glycosaminoglycans, chondroptosis, lipid droplets, and chondron counts were considered as our comparison criteria in our study.

In the comparison between 4 group of study, our experimental group showed least chondron length with mean difference of 55.250μm, 13.250μm and 94.750μm from main control, NPRS and Positive control group respectively. These findings alongside with much more round-shaped and polygonal geometry of chondron in the experimental group than others are showing that adding PRMS to chondrocytes can improve the chondrogenesis behavior of the OA-Chondrocyte which normally behaved as a formation of the more elongated chondrons with bizarre shape in main control group. The most bizarre and elongated chondron is related to our positive control group which we inspect multipolar and elongated chondron morphology in them.

The reason for chondron elongation in chondrocyte culture can be due to the cytoskeletal changes due to the possible inflammation pathway process (as we enhance it in positive control group or normally seen in OA-chondrocyte culture-main control group).

Chondron count also was shown a huge difference between the experimental group and other groups, by mean difference of 138.000, 99.500, 149.750 from main control, NPRS and positive control group respectively. Its quite clear that adding PRMS induced the chondron amount in the cell culture, hence its shown to have a great ability for accomplishing the proliferation-related part of our chondrogenesis hypothesis.

Our staining also showed the great ability of PRMS to enhance the precellular and Intraterritorial matrix production, as we have increased staining of related parts with Sirius-Red and Alician Blue which shows the presence of collagen, chondroitin and keratan sulfate respectively. We used Hydrogen chloride as a dye solvent for the purpose

of targeting the strong mucopolysaccharides structure of the intraterritorial matrix as keratan and chondroitin sulfate. Additionally, Alician Blue showed the characterization of chondrocyte as well. We observe most intensive staining with Alician Blue dye in our experimental group, therefor we can reveal that PRMS can induce the keatan and chondroitin sulfate production as well which show to be in lesser amount in other groups. Chondroitin and Keratan Sulfate are two important GAGs which have been considering by one of the important factors between scientist to evaluate the phenotypic behavior of the chondrocyte, especially in OA studies.

Neither of the groups got Alizarin Red dye and we could not see and stained chondrons. This revealed that, non of the groups shown the ossification signs in the culture plates. The ossification process has been generally monitored by Mesenchymal stem cell cultures when it tried to be differentiated to chondrocyte, in the purpose of evaluating the osteoblastic activity.

We also analyzed our color value density for Sirius Red staining to quantitatively analyze our collagen staining process. We create a color histogram for each chondron, and got the mean histogram color value level from NIH J Image software. Less parametric values in our color assessments describes the higher intensity of the red color in the assessed structure. Hence, we found the highest intensity of color with the mean difference of 72.186917, 51.951000, and 83.067750 from main control, NPRS and positive control group respectively. These findings show the highest collagen production amount in the PRMS group which alongside Alician Blue staining can be considered an accomplishment of our hypothesis in terms of phenotypic chondrogenesis ability of PRMS.

The Chondroptosis phenomenon also inspected in other cultures rather than PRMS. The highest chondroptosis amount was related to positive control group. This finding also helps our hypothesis toward the chondrogenesis induction by PRMS as chondroptosis as another type of apoptosis is related to damaged cell behavior and its a not good finding in each kind of cell line.

Lipid droplet amount also has been considered as an evaluation criterion for the comparison. The reason for selecting this criteria is that since cells usually use these droplets for its energy consumption generally, cellular lipid droplets can be used as indicators of cell metabolic activity. Hence, higher cell metabolic activity can be a sign of higher mitotic activity as well. We found an abundance of black dots that were similar to the fatty droplet findings of other studies' light microscopically findings, in all groups except positive control groups which had the least amount of them. But, evaluation of the lipid droplet and its precise characterization should better to be done by other techniques such as TEM (Transitional Electron Microscopy) to achieve better results. Our findings were not showing any meaningful differences between groups upon this criterion.

Fetomaternal Microchimerism is a phenomenon that is deserved to be paid much more attention in the regenerative medicine field. But the approach to it should be wisely determined to avoid any confusing inference which may lead to false-negative or falsely positive results. In our study, it's quite clear that PRMS (Pregnant Rat Maternal Serum) has a positive effect on chondrogenesis in both mitotic and phenotypic expression manner. And this is easily inferable by comparison of cell or chondron count, Collagen amount and cell morphology between the groups, as our statistical analysis also showed the acceptable and meaningful P.Value for that(p.value=.001 for Chondron Color,0.000 for Chondron Count and 0.015 for chondron length). However, The main reason for this effect will be evaluated in our next studies to find the main factor of chondrogenesis stimulation in pregnant maternal serum. But considering this study results, definitely, it's some factors related to the pregnancy and pregnant mother that initiate or enhance the chondrogenesis which should be investigated furthered to being identified.

Until now there were lots of substances applied to stimulate the chondrogenesis invitro but few of them showed a meaningful effect on chondrocytes. Among them is TGF-Beta which shows the good cell size induction ability but not good in proliferative and morphological manner of the chondrogenesis (147). Using of TGF-Beta subfamily adjacent with other substances also were investigated by the scientists (Such as icariin (133) and other growth factors) but despite some of the good achievements, they have

been not accomplishing all the factors that it resembles a good chondrogenesis stimulation yet. In some other studies, coculturing of mesenchymal stromal cells (MSCs) with chondrocytes, reduces the loss of collagen VI and improves extracellular matrix production (148, 149). In a study, chondrocytes Cocultured with Stromal Vascular Fraction of Adipose Tissue showed to have ability of presenting More Intense Chondrogenic Characteristics by expressing higher amount of GAG (150). Direct Coculture of Human Chondrocytes and Synovium-Derived Stem Cells also studied by Tae Woo and his colleagues, which showed Enhancement of In Vitro Chondrogenesis by expression of more collagen and GAG (151).

Our studies show not only the good proliferation stimulation ability but also good cellular morphology and behavior of the chondrocytes among with higher extracellular matrix proteins, which all are good signs of chondrogenesis.

Even though the main identity of the main stimulant factor in our study has not yet defined but we can declare that pregnant individual's serum shows a good therapeutic ability in-vitro and can have considerable therapeutic potential if used invivo as well. If invivo studies confirm this idea as well, a new horizon for osteoarthritic diseases treatment can be introduced to the world that would be technically much easier, affordable, and accessible among other approaches to OA treatment, Or at least it may be used as an adjuvant therapy with other approaches to maximize their therapeutic effects.

The fetomaternal microchimeric enriched concentrates from blood can be applied to synovial capsule to enhance the regeneration process. The effectiveness and accessibility of this approach can be further being compared with approved other concentrates such as Stromal Vascular Fraction (SVF) and Bone marrow aspirate concentrate (BMAC) in terms of regeneration capacity. But a great ratio of chondrogenesis observed in our study can represent the promising future of this method in clinical approaches. On the other hand, the Pleuropatency expression of fetal microchimeric cells which has been approved in the literature may multiply this possibility that these concentrates may carry a huge potential for the regeneration process. Additionally, with the less invitro preparation work needed, the chance of it

would carry less chance of contamination and other risks related to moderate invitro preparation work. Apart from the mentioned advantages, it seems to be much more cost-beneficial to be used in clinic.

Our next phase of studies would be invivo approvement of this approach in the animal model and trying to find the exact identity of the stimulant factor.

6.CONCLUSION

Chondrogenesis ability of pregnant rat maternal serum on the human osteoarthritic cell was evaluated in our study. Results showing that PRMS(Pregnant Rat Maternal Serum) is able to induce the chondrogenesis in OA-Chondrocyte in vitro by increasing the cell (and chondron) amount, induction of better chondrocyte morphology, more collagen, and more glycosaminoglycan(Chondroitin and keratan sulfate) in comparison with NPRS(Non-Pregnant rat serum) and normal control group(in which no additional substance added but than normal complete media). These results show that PRMS is the truly stimulant of chondrogenesis, but which factor from PRMS is the direct reason and stimulator of chondrogenesis and whether its related to the fetomaternal microchimerism is something that scheduled to be discovered in our next phase of the study. However, pregnant individuals' maternal serum itself, could be a promising treatment approach for OA which should be confirmed furtherer by invivo studies as well.

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Annex-1. Ethics Committee Approval

SAKİ YENİLLİ DENEY HAYVANLARI ÜRETİM VE UYGULAMALABORATUVARI TİC. LMT. ŞTİ.
HAYVAN DENEYLERİ YEREL ETİK KURULU
HAYVAN DENEYLERİ YEREL ETİK KURULU KARARI

TOPLANTI TARİHİ : 19/08/2020
TOPLANTI NO : 05
DOSYA NO : 03
KARAR NO : 14

Ankara Yıldırım Beyazıt Üniversitesi/ Ortopedi ve Travmatoloji ABD dan Prof.Dr.Murat Bozkurt yürütücülüğünü yaptığı Ankara Yıldırım Beyazıt Üniversitesi/ Kas İskelet Sistemi ve Rejeneratif Tıp bölümünden Ali Torabi nin katıldığı

İn-vitro osteoartritik modelde hamile sıçan maternal serumu kondrojenez üzerine etkilerinin araştırılması adlı çalışmanın Saki Yenilli Deney Hayvanları Üretim Ve Uygulama Laboratuvarı Hayvan Deneyleri Yerel Etik Kurulu Yönergesine göre aşağıda belirtilen kapsamda yapılmasına oy birliği ile karar verilmiştir.

HAYVAN TÜRÜ : Wistar Albino Rat
HAYVAN SAYISI : 20
GEÇERLİLİK SÜRESİ : 19/08/2020 – 19/08/2021

ETİK KURUL ÜYELERİ			
ADI SOYADI	ÜNVANI	GÖREVİ	İMZASI
MUSTAFA GÜRGEN	VET.HEKİM	ETİK KURUL BAŞKANI	
SAKİ YENİLLİ	PROTEZ-ORTEZ TEKNİKERİ	BAŞKAN VEK.	
HÜSEYİN HAYRİ KERTMAN	BEYİN CERRAHI	İNVİVO ÇALIŞACAK ÜYE	
ERCAN ŞAHİN	BİYOLOG	SİVİL TOPLUM KURULUŞUNA KAYITLI ÜYE	
SELİM KOCA	TEKNİKER	SEKRETER	
EMRAH SATILMIŞ	EDİTÖR	SİVİL ÜYE	
BORA GÜRER	BEYİN CERRAHI	İNVİVO ÇALIŞACAK ÜYE	

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Annex2. CV

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