EVALUATION OF EFFECTIVENESS OF DIFFERENT BIOACTIVE AGENTS FOR TREATMENT OF OSTEOARTHRITIS WITH IN VITRO MODEL UNDER DYNAMIC MECHANICAL STIMULATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYŞEGÜL KAVAS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
ENGINEERING SCIENCES

SEPTEMBER 2007

Approval of the thesis:

EVALUATION OF EFFECTIVENESS OF DIFFERENT BIOACTIVE AGENTS FOR TREATMENT OF OSTEOARTHRITIS WITH IN VITRO MODEL UNDER DYNAMIC MECHANICAL STIMULATION

submitted by **Ayşegül Kavas** in partial fulfıllment of the requirements for the degree of **Master of Science in Egineering Sciences Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Turgut Tokdemir
Head of Department, **Engineering Sciences Dept.** _____

Assist. Prof. Dr. Ayşen Tezcaner
Supervisor, **Engineering Sciences Dept., METU** _____

Assist. Prof. Dr. Dilek Keskin
Co-supervisor, **Engineering Sciences Dept., METU** _____

Examining Committee Members:

Assoc. Prof. Dr. Hakan I. Tarman
Engineering Sciences Dept., METU _____

Assist. Prof. Dr. Ayşen Tezcaner
Engineering Sciences Dept., METU _____

Dr. Sreeparna Banerjee
Biological Sciences Dept., METU _____

Assist. Prof. Dr. Ergin Tönük
Mechanical Engineering Dept., METU _____

Assist. Prof. Dr. Dilek Keskin
Engineering Sciences Dept., METU _____

Date: 07.09.2007

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name :

Signature :

ABSTRACT

EVALUATION OF EFFECTIVENESS OF DIFFERENT BIOACTIVE AGENTS FOR TREATMENT OF OSTEOARTHRITIS WITH IN VITRO MODEL UNDER DYNAMIC MECHANICAL STIMULATION

Kavas, Ayşegül

M.S., Department of Engineering Sciences

Supervisor : Assist. Prof. Dr. Ayşen Tezcaner

Co-Supervisor: Assist. Prof. Dr. Dilek Keskin

September 2007, 84 pages

Osteoarthritis (OA) is a disease characterized by the progressive degradation of articular cartilage. Current strategies for the disease are mainly towards relieving symptoms. This study was aimed to investigate the therapeutic potentials of Bone Morphogenetic Protein-9 (BMP-9), Raloxifene (Ral) and Pluronic F-68 (PLF-68) with a three-dimensional *in vitro* OA model.

Articular chondrocytes isolated from rats were cultured in growth media and embedded in agarose to obtain agarose-chondrocyte discs. Dynamic hydrostatic mechanical stress was applied to discs. The discs were incubated with Aza-C for 48 hours for OA development. After its removal, chondrocytes were treated with different doses of BMP-9, Ral and PLF-68 for 10 days. The efficacies of treatments were evaluated by measuring cell number, glycosaminoglycan and collagen amount, and mechanical properties of the

discs. Measurements of these properties were performed with MTT, quantitative colorimetric assays, histochemical staining and mechanical tests, respectively.

According to comparative results with healthy groups and controls (osteoarthritic chondrocytes without any treatment), it was found that BMP-9 had negative effect on osteoarthritic chondrocytes. On the other hand, Ral showed positive results related with matrix synthesis and mechanical properties especially at 5 μM dose suggesting that it holds promise for the treatment of OA. The therapeutic effect of Ral on OA was documented for the first time in literature. The potential of PLF-68 for treatment of OA was also supported by this study considering its positive effects on cell number, collagen synthesis and mechanical properties. Yet, further investigations are also suggested for conclusive results on this agent.

Keywords: Osteoarthritis, Bone Morphogenetic Protein-9, Raloxifene, Pluronic F-68, agarose

ÖZ

DİNAMİK MEKANİK STİMULASYON ALTINDAKİ İN VİTRO MODELDE FARKLI BİYOAKTİF AJANLARIN OSTEOARTRİT TEDAVİSİNDE ETKİNLİKLERİNİN DEĞERLENDİRİLMESİ

Kavas, Ayşegül

Yüksek Lisans, Mühendislik Bilimleri Bölümü

Tez Yöneticisi : Y. Doç. Dr. Ayşen Tezcaner

Ortak Tez Yöneticisi: Y. Doç. Dr. Dilek Keskin

Eylül 2007, 84 sayfa

Osteoartrit (OA) eklem kıkırdağının ilerleyen bozunması olarak tanımlanmaktadır. Hastalık için günümüzde kullanılan stratejiler temel olarak semptomları azaltmaya yöneliktir. Bu çalışmada Kemik Morfojenik Protein-9 (KMP-9), Raloksifen (Ral) ve Pluronik F-68 (PLF-68)'in tedavi potansiyellerinin üç boyutlu *in vitro* OA modelinde araştırılması amaçlanmıştır.

Sıçanlardan izole edilen eklem kıkırdak hücreleri büyüme ortamında çoğaltıldı ve kondrosit-agaroz diskler elde etmek için agaroza gömüldü. OA oluşumu için diskler 48 saat süreyle Aza-C ile inkübe edildi. Aza-C uzaklaştırıldıktan sonra, kondrositlere 10 gün boyunca KMP-9, Ral ve PLF-68'in farklı dozları uygulandı. Disklere dinamik hidrostatik mekanik mukavemet uygulandı. Tedavilerin etkileri disklerin hücre sayısı, glikozaminoglikan ve kolajen miktarı ile mekanik özellikleri ölçülerek

değerlendirildi. Bu özelliklerin ölçümleri sırasıyla MTT, sayısal kolorimetrik metotlar, histokimyasal boyama ve mekanik testlerle yapıldı.

Sağlıklı grup ve kontrollerle (herhangi bir tedavi görmemiş osteoartritik kondrositler) karşılaştırmalı sonuçlara göre, KMP-9'un osteoartritik kondrositler üzerinde olumsuz etkisi olduğu bulunmuştur. Diğer yandan, Ral OA'in tedavisi için umut vadettiğini gösteren, özellikle 5 μM'lık dozda matris sentezi ve mekanik özellikler bakımından olumlu sonuçlar göstermiştir. Ral'in OA üzerinde tedavi etkisi yazılı kaynaklarda ilk defa belgelenmiştir. PLF-68'in hücre sayısı, kolajen sentezi ve mekanik özellikler üzerindeki olumlu etkileri göz önüne alındığında OA'in tedavisi için potansiyeli olduğu bu çalışmayla desteklenmiştir. Fakat, bu ajan ile ilgili sonuçlar üzerinde farklı araştırmalar yapılması önerilmektedir.

Anahtar kelimeler: Osteoartrit, Kemik Morfojenik Protein-9, Raloksifen, Pluronik F-68, agaroz

To My Family

ACKNOWLEDGEMENTS

I would like to present my most intensive gratitude to my supervisor Assist. Prof. Dr. Ayşen Tezcaner and co-supervisor Assist. Prof. Dr. Dilek Keskin for their continuous support, guidance, encouragement, and advice throughout this study.

I would like to thank Prof. Dr. M. Ruşen Geçit for his valuable support, suggestions and encouragement during this study.

I wish to extend my thanks to Assist. Prof. Dr. Senih Gürses for his advice, support and solutions he offered in some critical points during this research.

I would like to present my thanks to Dr. Sreeparna Banerjee for providing the use of hot plate and high-speed centrifuge in the experiments.

I would like to thank Assoc. Prof. Dr. Çetin Kocaefe for his cooperation in crossectioning the agarose discs for histological analysis.

I wish to thank Biolab Ltd, Turkey for providing custom-made pressure chamber which is an essential component of this study.

I would like to present my acknowledgements to my friend Özge Erdemli for her friendship, valuable help in the experiments and enjoyable time we shared together making the long hours shorter.

I wish to express my thanks to my labmates Burçin Başar, Ömer Aktürk, Özlem Aydın, Parisa Sharafi and Seylan Aygün for their friendship and support during this research.

I would like to present my thanks to my friends Beril Korkmaz Erdural, Deniz Alev Öztürk, Emel Ortalan, Ersan Güray, Özlem Ak, Seçil Sanıyaman, Tijen Seyidoğlu and Volkan İşbuğa for their valuable support and sincerity during this study.

I would like to thank my family for their continuos support, trust in me, encouragement and understanding throughout this study. I feel myself so lucky.

I would like to thank The Scientific and Technological Research Council of Turkey (Project Number: TBAG 104T 496) for the support they provided.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

3-D	Three-dimensional
Aza-C	5-Azacytidine
BMP-9	Bone Morphogenetic Protein-9
COMP	Cartilage oligomeric matrix protein
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modifed Eagle's Medium
ECM	Extracellular matrix
FCS	Fetal calf serum
IL-1β	Interleukin-1β
KS	Keratan sulfate
LDL	Low-density lipoprotein
LOX-1	Lectin-like oxidized LDL receptor-1
MMPs	Matrix metalloproteases
NO	Nitric oxide
OA	Osteoarthritis
PLF-68	Pluronic F-68
Ral	Raloxifene
SEM	Scanning electron microscope
SERM	Selective estrogen receptor modulator
sGAG	Sulfated glycosaminoglycan
TCPS	Tissue culture grade polystyrene
TGF-β1	Transforming growth factor-β1
Trypsin-EDTA	Trypsin-ethylenediamine tetraacetic acid

CHAPTER 1

INTRODUCTION

1.1. Articular Cartilage

The articular cartilage is a shiny, slippery, pearly-blue-white tissue that grossly covers the articulating ends of the bone. It is an avascular tissue that contains only one cell type, chondrocyte, and has a very limited capacity for self-repair (Figure 1.1.) (Kuettner et al., 1991). Each chondrocyte in the articular cartilage is isolated to its own lacuna and is surrounded by a prominent proteoglycan bluish ring. Chondrocytes are not arranged in groups or clusters.

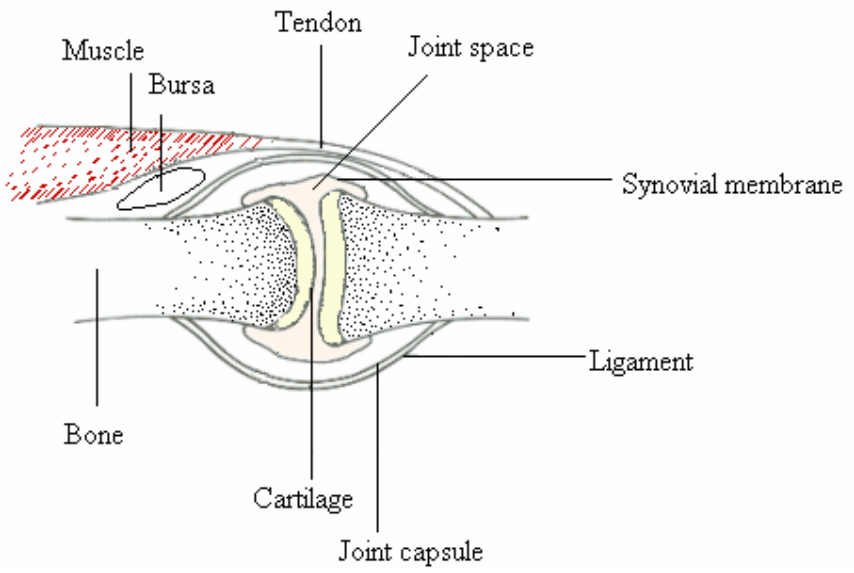


Figure 1.1. Anatomical structure of an articular joint.

The chondrocytes are responsible for the synthesis and maintenance of their ECM, which is composed of a hydrated collagen network (~ 60 % of the tissue dry weight), a highly charged proteoglycan gel (PG, ~ 25 % of the tissue dry weight), and other proteins and glycoproteins (~ 15 % of the tissue dry weight). Its high water content (70 to 80 % of the tissue wet weight) enables cartilage to withstand the compressive, tensile, and shear forces associated with joint loading (Kuettner et al., 1995).

The normal synovium is richly vascularized; soft, with some villi, and confined to the lateral ends of the joint. It never totally covers nor adheres to the articular cartilage. Microscopically, the synovium is composed of two zones: a synovial lining layer that is one or two cells (synoviocytes) thick without a basement membrane and a deeper layer of supporting tissue, either fat or loose fibrous tissue with a prominent vascular supply. The synovial cells have secretory and phagocytic functions (Kuettner et al., 1995). In synovial joints, the layer of hyaline articular cartilage tissue faces the joint cavity (i.e. the synovial fluid space) on one side and is linked to the subchondral bone plate via a narrow layer of calcified cartilage tissue on the other (Kuettner et al., 1995).

Microscopically, normal articular cartilage is composed of three zones that are based on the shape of chondrocytes and distribution of the type II collagen: the tangential or superficial zone, the intermediate zone, and the basal zone (Figure 1.2.). The tangential zone has flattened chondrocytes, tangentially arranged and condensed collagen fibers, and relatively sparse proteoglycan. In the intermediate layer, the chondrocytes are round and tend to be oriented in perpendicular or vertical columns paralleling the collagen fibers. The basal layer is the deepest layer, and in it the chondrocytes are round. Deep to the calcified cartilage and supporting it is a layer of lamellar bone called the subchondral bone plate (Figure 1.2.) (Kuetner et al., 1995).

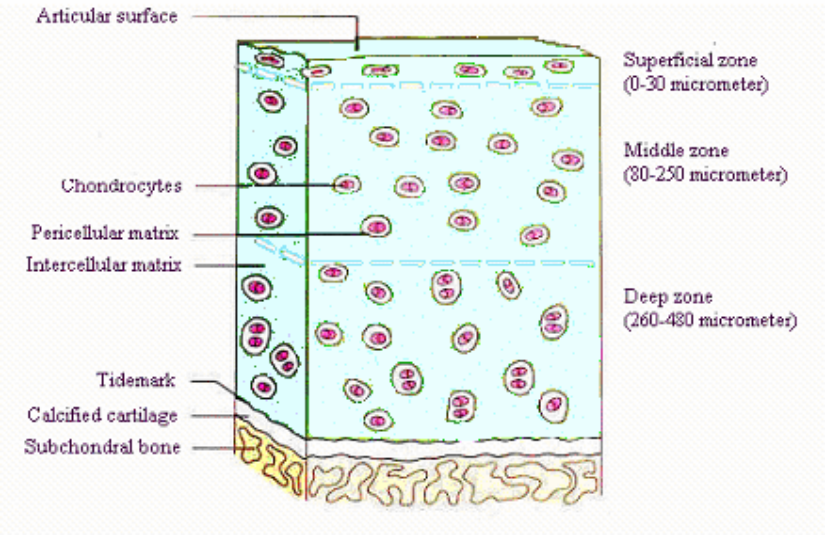


Figure 1.2. Schematic drawing of articular cartilage.

1.1.1. Diseases Affecting the Articular Cartilage

Arthritis means the inflammation of a joint. Although inflammation is a sign of trouble, it is actually a renewing process that occurs in response to injury of living tissue. It is a positive healing process if it does not become chronic. It is the chronic nature of arthritic inflammation that causes chain reactions leading to arthritic symptoms (such as joint pain, tenderness, warmth, swelling, and redness) and complications (Gordon NF, 1993).

Arthritis is divided into two main forms: i) the atrophic form characterized by synovial inflammation, generalized loss of cartilage, and bony erosions (i.e. rheumatoid arthritis), ii) the hypertrophic form characterized by focal areas of cartilage damage, overgrowth of subchondral and marginal bone, and thickening of the capsule (i.e. osteoarthritis). Although both diseases result in loss of joint function, the mechanism behind the cartilage degeneration is completely different for these diseases.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and abnormal immune reactions resulting in cartilage destruction, bone erosions, periarticular osteoporosis, and generalized bone loss (Seemayer et al., 2005). Activated cells of the synovium produce pro-inflammatory cytokines and matrix degrading enzymes that sustain the inflammation and permanent joint damage (Gay et al., 2002, Ospelt et al., 2004, Seemayer et al., 2005). These processes result in increased prevalence of osteoporotic fractures. Some of the disease mechanisms responsible for focal bone loss may be similar to the processes of generalized osteoporosis and hence be associated with osteoclast activation (D'Elia et al., 2004). The initial events resulting in development of RA have not been completely understood. Osteoarthritis (OA), a second prevalent arthritic disease, will be discussed in more detail in the following sections.

There is an increasing prevalence of arthritic diseases like RA and OA in today's human society. This has led to a growing demand in development of new and safer treatments since these diseases abolish the quality of patients' lives.

1.1.1.1. OA: One of the World's Most Incident Joint Disease

The global impacts of OA on function and costs of care are substantial. OA can be characterized by progressive articular cartilage loss, narrowing joint space, formation of osteophytes, subchondral bone fractures, and varying degrees of mild chronic inflammation of the synovium which lead to chronic pain and functional restrictions in the affected joints (Lorenz and Richter, 2006, Nesic et al., 2006). Fortunately the damage is limited to the musculoskeletal system and it usually involves only one or a few joints. The weight bearing joints-feet, knees, hips and the spine-as well as the digital joints of fingers, hands and toes are most likely to be the problematic areas (Gordon, 1993).

Mostly traumatic events are the causes of the development of OA, but genetic factors, defective position of joints; ageing and malnutrition are among the factors that can lead to similar alterations in the joint cartilage (Swoboda, 2001). Older aged people are among the highest risk groups for OA development. Obesity should also be indicated as a possible risk factor for the development of knee, hip and perhaps hand OA development due to increased joint loading (Chen et al., 2006, Felson et al., 1988, Oliveria et al., 1999, Karlson et al., 2003).

Experimental studies suggest that lipid peroxidation is involved in the pathogenesis of articular cartilage degradation. Hypertension and hypercholesterolemia characterized by the decreased removal of LDL are pronounced among the high risk factors. Akagi and his coworkers (2006) observed colocalization of chondrocytes associating with oxidized LDL and expressing LOX-1. LOX-1 is a member of the natural killer receptor gene complex found in endothelial cells**.** The group found significant correlations between the grade of cartilage degradation and the immunoreactivity of oxidized LDL and LOX-1. In conclusion, they showed that oxidized LDL reduced the human chondrocyte viability and proteoglycan synthesis, and pretreatment with anti-human LOX-1 monoclonal antibody reversed these effects.

The prevalence of OA in all joints correlates strikingly with age. With the exception of disease in the hip, OA is more seen in women than in men above the age of 50. (Kuettner, 1991). Sex and age-related prevalence patterns are consistent with the role of postmenopausal estrogen deficiency in increasing the risk of OA. Both incidence and prevalence appear to level off or decline in both sexes at around age 80 (Felson and Zhang, 1998).

1.1.1.1.1. **Pathophysiology of OA**

Typical features of OA are the degeneration or progressive loss of the structure and functionality of the articular cartilage due to the imbalance between anabolic and catabolic processes in the tissue. The precise mechanism of articular breakdown of cartilage in OA is still unclear. Several studies showed that the high rate of chondrocyte apoptosis occurs after mechanical trauma, release of cytokines and nitric oxide. Apoptosis has been shown to be linked with mechanical trauma, proteoglycan depletion from ECM and calcification in the cartilage layer (Patwari et al., 2003, Piscoya et al., 2005, Hashimoto et al., 1998, Gannon et al., 1999).

The progression of OA is generally divided into three broad stages, namely the proteolytic breakdown of the cartilage matrix, the fibrillation and erosion of the cartilage surface, and the beginning of the synovial inflammation (Martel-Pelletier, 2004). Due to the limited regenerative capability of AC, the progression of this degenerative joint disease has to be detected before irreversible morphological changes become evident. The repair tissue that forms in response to cartilage loss is mostly fibrous and its mechanical properties are relatively weak compared to healthy hyaline cartilage. Early diagnosis of OA will enable an early treatment, the reduction of pain and disability and thus the improvement of the quality of patient's life.

At the beginning of the degeneration process the cartilage surface slightly changes and the matrix network degrades at the molecular level. There is an increased water deposition within the cartilage in the early, hypertrophic phase of OA (Appleyard et al., 2003). Hypertrophic phenotype of cartilage is thus an indicator of increased metabolic activity of these cells associated with the repair process. Mild fibrillations are also evident in the cartilage tissue. However, there is no change in the homogeneous distribution of glycosamino-

glycans. Several studies in different animal models and human studies showed increase in the level of MMP13 (Bluteau et al., 2001, Lorenz et al., 2005, Tchetina et al., 2005). MMP13 is one of the matrix metalloproteases that is upregulated in OA and is responsible for severe cartilage degradation. In the advanced stages of OA, fibrillated areas containing collagen type I become more abundant while deposition of collagen type II (characteristic cartilage ECM collagen) decreases. The distribution of matrix components is altered which demonstrates their breakdown (Lorenz and Richter, 2006).

Local inflammatory processes are accompanied with the upregulation of cytokines IL-1β and TNFα that have been shown to contribute the pathological development of OA. IL-1 and TNFα increase nitric oxide synthase leading to high nitric oxide level which results in downregulation of matrix synthesis and upregulation of matrix degradation via activation of MMPs (Murrel et al., 1995, Studer et al., 1999). Additionally, increase in NO results with susceptibility of chondrocytes to oxidants and apoptosis of these cells (Blanco et al., 1995). The degradation of aggrecan, the second most prevalent ECM component, is mediated by aggrecanases (Arner, 2002). Although the initial inducers of cartilage catabolism in OA are not yet identified, potential stimuli of these are mainly mechanical stress (Patwari et al., 2003, Piscoya et al., 2005) and degradation products of ECM (Forsyth et al., 2002, Homandberg et al., 1996, Stanton et al., 2002). Fibronectin fragments induce the synthesis of MMPs which degrade collagens, elastins and other ECM components. Il-1 is a proinflammatory cytokine in OA degradation. Unlike the case with rheumatoid arthritis and other inflammatory arthritis, inflammation of OA is usually mild and localized to the affected joint.

Age related changes are structural and biochemical matrix organization, surface fibrillation, alteration in proteoglycan composition, increased collagen crosslinking, cumulative oxidative damage and accumulation of mutations and

genetic instability (Nesic et al., 2006). These are all interdependent mechanisms that decrease functionality of cells with age and lead to cell senescence.

1.1.1.1.2. Treatments in Medicine

OA is generally diagnosed in more advanced stages, when clinical and radiographic signs become evident. At this stage, however, the options for therapeutic intervention without surgery are limited. The clinical management of OA involves control of pain, improvement in function and in health-related quality of life.

The recommended approaches to the medical management of hip or knee OA include non-pharmacologic modalities, drug therapy and surgery. Drug therapy for pain management is most effective when combined with non-pharmacologic strategies like therapeutic exercise alone, ice massage, joint bracing, and weight loss. Pharmacologic therapies for short term include intraarticular injections of opioids, glucosamine sulfate, chondroitin sulfate hyaluronic acid, acetaminophen, selective and nonselective NSAIDs (non-steroidal anti-inflammatory drugs).

Chondroitin sulfate-hyaluronic acid are now seen as disease modifying agents as a result of animal, *in vitro* and short run clinical studies (Glass, 2006). However, in a study carried out to observe the effects of intraarticular hyaluronan injection period on treatment of knee OA, conflicting results were obtained. Finally, according to animal studies, Brandt et al. (2000) suggested that although intraarticular injection of hyaluronan therapy seems safe in the short-run, it might cause overloading of the damaged joint, leading to depletion of proteoglycans in articular cartilage and increase in structural damage in the long-run.

Clinical trials showed that glucosamine, mostly used for relief from disease symptoms, has a moderate treatment effect, whereas chondroitin sulfate has a larger treatment effect (Leeb et al., 2000, McAlindon et al., 2000, Richy et al., 2003). Additionally, Homandberg and his coworkers recently (2006) showed that mixtures of glucosamine and chondroitin sulfate acted synergistically and reversed fibronectin fragment mediated damage to cartilage more effectively than either agent alone.

Obesity is also seen as one of the risk factors for OA development (Oliveria et al., 1999, Karlson et al., 2003). Contradictory results were shown by another group's recent work on the protective role of adinopectin in OA, a hormone released by adipocytes (Chen et al., 2006). They studied the effects of adiponectin on primary chondrocyte functions and found that this hormone down regulated IL-1β-induced MMP13. These findings indicated that adiponectin could act as a protective role in the progression of OA. Collectively, the authors concluded that further studies are needed to understand the molecular mechanisms responsible for the decrease. Previous reports also indicated that the mean synovial fluid adinopectin levels of OA patients were lower than the RA patients. Additionally, a negative correlation was found between synovial fluid adinopectin levels and severity of OA. However, obesity should remain as a risk factor for OA due to a leading cause for overloading the joints.

The degenerative changes of OA can alter joint mechanics. Patients who continue to have severe pain and functional limitation despite the pharmacological and non-pharmacological therapy are considered for surgical treatment. Surgical treatment of OA aims decreasing or eliminating pain and improving function. To date, the treatment of OA consists of either obliteration of the joint by joint fusion (arthrodesis) or replacement of the joint with an artificial bearing by pressure reducing surgery such as muscle lengthening.

Joint fusion, although functional, can be awkward to the patient and may be related to eventual OA in the neighboring joints, which are obviously under greater stress. Joint replacement, although providing excellent short- and medium-term relief while maintaining functional range of joint motion, has poor long-term results in functionally active patients, and subsequent revision has been shown to have an even shorter life span (Kuettner, 1991). There are also surgical procedures performed to supply bone marrow derived mesenchymal precursor cells to the damaged site by penetrating to the underlying subchondral bone. These procedures, however, result in fibrocartilage, not articular cartilage (Suh et al., 1997, O'Driscoll, 1999).

Different cell and biomaterial based tissue engineering strategies (delivery of cells and growth factors for regeneration) are also under investigation to restore damaged cartilage due to inability of adult to self-heal cartilage damage (Robert et al., 2003, Mizuta et al., 2004, Na et al., 2006, Mouw et al., 2005). This is primarily because of the absence of mitotic activity of differentiated chondrocytes, a minimized supply of nutrients in the joint (with diffusion) and possibly a low concentration of mesenchymal stem cells. In contrast, cartilage injuries in children show a good capacity to self-heal as a result of a higher stem cell concentration.

Cartilage regeneration using cell based tissue engineering has been shown as a promising strategy. The first clinical application involves autologous cell implantation (ACI) after multiplication of autologous chondrocytes *in vitro*. For functional cartilage tissue engineering polymeric three-dimensional carriers (porous supports, injectable, bead form etc.) that are mainly hydrogel are designed for both cartilage and osteochondral defects (Wakitani et al., 2002, Sherwood et al., 2002, Tamai et al., 2005, Li et al., 2006, Oliveria et al., 2006). Among these are agarose, fibrin, collagen, hyaluronan, alginate, alginate-gelatin blends, polyglycolic acid (Mauck et al., 2000, Démartau et al.,

2003, Mouw et al., 2005, Na et al., 2006, Schagemann et al., 2006, Eyrich et al., 2007, Lee et al., 2007, Chen et al., 2007).

Regeneration of human articular cartilage defects in osteoarthritic knees was promoted by autologous mesenchymal stem cell transplantation. Autologous mesenchymal stem cells were isolated from the bone marrows aspirated from both sides of the iliac crests of 24 patients (Wakinati et al., 2002). These stem cells were embedded in collagen gels, and then transplanted into the articular cartilage defects which were covered with the periosteum. At the end of 42 weeks cartilage-like tissue was observed. The arthroscopic and histological evaluations were found better than the cell-free control group. This study showed that mesenchymal stem cells found in bone marrow could be an alternative cell source for cartilage repair (Murphy et al., 2003, Mizuta et al., 2004). Tissue engineered constructs with allogeneic chondrocytes are on the market and find widespread use clinically. Such engineered cartilage constructs not only serve as grafts to promote repair of large joint injuries but also serve as model systems for controlled studies of cartilage development and diseases mechanisms.

1.2. Research on Treatment of OA

1.2.1. OA Models

Experimental models for OA can be subdivided into two: *in vitro* and *in vivo* models (animal models). *In vitro* models can be cartilage explants (Piscoya et al., 2005, Homandberg et al., 2006), two- or three-dimensional growth of chondrocyte cells (osteoarthritic cells isolated or induced osteoarthritic cells) with or without carrier systems (pellet co-cultures, monolayer growth) (Dodge and Jimenez, 2003, Lübke et al., 2005). *In vivo* models, however, involve formation of OA in animal models either by surgery (i.e. meniscectomy and transection of the anterior cruciate ovarectomy of the female animals) (Roos et

al., 1995, Høegh-Andersen, 2004, El Hajjaji, 2004) or intraarticular injection of agents (i.e. collagen, fibronectin fragments, papain) (Homandberg et al., 1993, Homandberg et al., 2001). Either *in vivo* or *in vitro* models aim to study the mechanisms for the development and progression of OA and to test the therapeutic or preventive effectiveness of potential agents (glucosamine, chondroitin sulfate, adiponectin, etc.) (Dodge and Jimenez, 2003, Homandberg et al., 2006, Chen et al., 2006).

1.2.1.1. *In Vitro* Models

Apart from development of new regenerative or preventive therapy approaches establishment of *in vitro* models stimulating certain pathophysiological processes in diseases like RA and OA receives attention among researchers to understand the mechanism of the disease.

The pellet culture model has been used for studying the mechanisms of chondrogenesis by many researchers (Yang et al., 2004, Tallheden et al., 2004). Cartilage cells after low speed centrifugation do not adhere to the walls of the centrifuge tube, and form an essentially spherical aggregate. It was shown that the chondrocytes redifferentiate and form a cartilage-like tissue even after monolayer culturing for a long time. However, such systems were not used as model systems for osteoarthritic model development. They were used as an alternative interactive culture system *in vitro* model for rheumatoid arthritis (Lübke et al., 2005). Lübke and his coworkers co-cultured the porcine chondrocyte pellet culture with synovial fibroblast cell line derived from rheumatoid arthritis patients. Human origin synovial fibroblasts showed cartilage invasion as it occurs *in vivo* in RA. On the other hand, some researchers seed osteoarthritic cells at a high density and grow them as a monolayer for their OA studies (Bobacz et al., 2004, Chen et al., 2006, Akagi et al., 2007) in order for the cells not to dedifferentiate.

Cartilage plugs (explants) maintained in stable and controlled biochemical and physical environments are most preferred models to study the mechanisms underlying cartilage degenerative diseases and therapeutic efficacy of drugs. Explant cultures are obtained by taking full thickness biopsy from the cartilage. This provides the benefit of studying an intact tissue. The cells retain their characteristic phenotype, spatial arrangement and differentiated state as *in vivo*. Piscoya and his coworkers (2005) studied the effects of mechanical compression over normal physiological range on the induction of production and release of osteoarthritis related biological markers. Homandberg and his group (2006) established an OA model with fibronectin-mediated damage on cartilage explant and studied protective roles of glucosamine, chondroitin sulfate and their mixtures. The group showed that their mixtures have better reversal effect on damage and positive effect on the repair. However, OA induction in explant models is also achieved with mechanical trauma (Rundell et al., 2005, Huser et al., 2006).

In vitro three dimensional OA models have also been developed embedding isolated chondrocytes in polymeric scaffolds (collagen, alginate, etc.) to investigate the pathways of OA development and the mechanism of the potential anti-osteoarthritic drugs (Cortial et al., 2006, Ho et al., 2006). The advantage of 3-D *in vitro* models compared to growing the chondrocytes as a monolayer in tissue culture flasks is to mimic their microenvironment as close as possible to *in vivo* conditions preventing major changes in cartilage phenotype.

Artificial degradation through enzyme treatment (i.e. trypsin, collagenase) (Nieminen et al., 2002, Korhonen et al., 2003, Rieppo et al., 2003, Moody et al., 2006), pro-inflammatory cytokines (i.e. IL-1) and apoptosis-inducing agents (azacytidine) (Ho et al., 2006) have been shown to produce models that resemble different stages of OA.

Artificial degradation using enzymes like trypsin, results in the loss of collagen fibrils and proteoglycans. One advantage for such models is that the degradation can be done under controlled conditions. Moody et al. (2006) used a proteoglycan based model system to resemble early stage OA. The group concluded that the consistency of trypsin treatment regimes for model development depends on the initial concentration, distribution and depth of the proteoglycans.

Ho et al. (2006) established a cell culture model mimicking the terminal differentiation that occur in osteoarthritic chondrocytes. For that purpose they treated the chondrocytes cells embedded in alginate beads with an apoptotic agent, 5-azacytidine (Aza-C). They observed both phenotypic and genetic changes of articular cartilage as a decrease in collagen type II expression and increase in collagen type X and ALP, markers for hypertrophic chondrocytes and besides increased apoptosis rate (20 %) as compared to control groups.

Another group embedded freshly isolated calf articular chondrocytes in collagen based scaffold and used different doses of IL-1 (0.1-20 ng/ml) to activate the expression of several matrix metalloproteinases (MMPs) that destroy ECM proteins like type II collagen and aggrecan (Cortial et al., 2006). They found that the concentration of IL-1 should be at least 1 ng/ml to observe significant phenotypic and gene expression changes related with the establishment of an *in vitro* OA model.

1.2.1.2. *In Vivo* Models

Animal models are frequently used in orthopedic researches to investigate effects of traumatic injuries on articular cartilage *in vivo*, to study the pathological variation in disease progression or to evaluate the potential of disease-modifying drugs. Well-defined time course, the easier access to the

joint and tissues enabling the monitor of the disease progression with several quantitative methods are the advantages of the use of animals in OA studies. Limitations are ethical issues, high costs, slow time course with large animals and the physiological and anatomical differences between animals and human.

Spontaneous OA animal models occur quite sporadically in knee joints of various strains of mice, in bovine and guinea pigs due to unknown causes of the onset of the disease. Transgenic and knockout mice (i.e. mice with inactivated type II procollagen) are more reliable and facilitate studying the role of specific mediators in the pathogenesis of OA and mimicking different stages and forms of osteoarthritic changes in articular cartilage (Hyttinen et al., 2001).

Experimentally induced OA models are divided into a chemical (intraarticular injection chemical reagents and biological mediators) and physical type (surgical) (Brandt, 2002). Among the agents used for OA development include the enzymes (i.e. papain, collagenase) (Scheck and Sakovich, 1972, Yeh et al., 2007) and degradation products of ECM components (i.e. fibronectin fragments).

The surgical induction is mainly performed in larger animals such as dogs, cats and rabbits. Common methods are menisectomy and transection of the anterior cruciate ligament (ACLT), which both result in true instability of the joint.
These surgical induction methods mimic OA progression naturally occurring in humans after traumatic injuries (Roos et al., 1995). However, the progression of OA-like changes in cartilage in physically induced *in vivo* models is slower compared to chemically induced ones. A specific OA animal model is the joint immobilisation of canine knees (Leroux et al., 2001, Vanwanseele et al., 2002). In such models, changes in mechanical, biochemical and morphological properties of articular cartilage was observed.

Overall, when the results from spontaneous osteoarthritic changes *in vivo* (from human cadaver specimens) were compared to animal models in terms of decrease in mechanical properties and sGAG content, and increase in water content, similar changes were observed.

1.2.2. Candidate Therapeutic Agents for OA Treatment

1.2.2.1. Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are multifunctional molecules belonging to the TGF-β superfamily. They are both growth and differentiation factors that were originally detected in and purified from demineralized bone (Majumdar et al., 2001).

The activity of BMPs was first identified in the 1960s (Urist, 1965), but the proteins responsible for bone induction remained unknown until the purification and sequencing of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and -4 in late 1980s (Wozney et al., 1988, Luyten et al., 1989, Wozney, 1992). Around 20 BMP family members have been identified so far. They have been shown to function as key regulators in cartilage and bone development during embryogenesis as well as in repair and remodelling processes of the adult skeletal system (Majumdar et al., 2001). The positive effects of BMPs -2, -4, -7, -9 and -13 on matrix synthesis and maintenance of cartilage phenotype were also shown by some researchers (Luyten et al., 1994, Flechtenmacher et al., 1996, Erlacher et al., 1998, Sailor et al., 1996, Weisser et al., 2001, Hills et al., 2005, Yeh et al., 2007).

Cartilage has a limited capacity to heal. Although chondrocyte transplantation is a useful therapeutic strategy, the repair process can be lengthy as discussed in tissue engineering approach in treatment section. The stimulatory effects of BMP-7, apart from osteo-inductive capacity, on cartilage growth, maturation

and matrix synthesis (aggrecan, collagen type II) in bovine and human chondrocyte cultures were documented (Nishida et al., 2000, Chubinskaya et al., 2003, Nishida et al., 2004). Hidaka and his coworkers (2003) have previously shown that over expression of bone morphogenetic protein-7 (BMP-7) in chondrocytes by adenovirus-mediated gene transfer has led to increased matrix synthesis and cartilage-like tissue formation *in vitro*. BMP-7 was also shown to accelerate the appearance of hyaline-like repair tissue in experimental cartilage defects. By this way, rehabilitation after cell-based cartilage repair could be prolonged, leading to increased patient productivity and life quality (Hidaka et al., 2003). Recently, Stöve and his coworkers (2006) showed that BMP-7 has anabolic stimulatory effect on human osteoarthritic chondrocytes embedded in alginate beads in terms of increased proteoglycan production and aggrecan expression.

In the study targeted to analyze the effect of bone morphogenetic proteins on chondrogenesis, the effect of proinflammatory cytokine interleukin-1 on chondrogenic-differentiated cells and the interaction of IL-1β with bone morphogenetic proteins were investigated. It was found that bone morphogenetic proteins -2 and -9 induced expression of type II collagen mRNA and increased expression of aggrecan and cartilage oligomeric matrix protein in cultured chondrocytes. This has also suggested that both growth factors induced the chondrogenic differentiation of the cells. Moreover, it was found out that bone morphogenetic proteins were able to partially block the chondrogenesis suppression effect of interleukin-1. Therefore, it can be concluded that bone morphogenetic proteins play an important role in chondrogenesis and have great potential as therapeutics in cartilage repair (Majumdar et al., 2001).

Additionally, the effect of BMP-9 on metabolism of juvenile and adult bovine cartilage was studied *in vitro*. In the same study, the effect of BMP-9 was also compared with those produced by two previously characterized BMPs: BMP-2

and 13 (CDMP-2). It was shown that BMP-9 led to increased stimulation of both proteoglycan and collagen synthesis in juvenile cartilage while BMP-2 provided an increase only in proteoglycan synthesis but not collagen synthesis. As a conclusion, the authors concluded that BMP-9 treatment lead to a significant reduction in the turnover rate of proteoglycans in juvenile explants, whereas all three BMPs were unable to induce a measurable anabolic response in adult cartilage explants (Hills et al., 2005).

1.2.2.2. Surfactants

Clinical studies of traumatic joint injury often document early pain and development of chronic diseases, such as OA. The chronic diseases can be initiated by cell death that occurs in articular cartilage during mechanical trauma to the joint. Main feature of cellular death by necrosis is swelling due to inability of cells to maintain ionic gradients across a damaged cell membrane. The cell eventually ruptures.

One group of substances much used in the physical sciences as boundary lubricants and for transforming hydrophilic subphases to hydrophobic surfaces are surfactants. Moreover, surface-active phospholipid, known as a surfactant in the lung, is present in the synovial fluid of normal joints in appreciable quantities. These small molecules bind to amino acid groups that comprise the protein chains in proteoglycans such as lubricin (Hills et al., 2003).

Poloxamer 188, P188 (Pluronic F-68, PLF-68, Sigma) is a water soluble triblock copolymer of poly(oxyethylene) and poly(oxypropylene) (POE-POP-POE). The POE chains are hydrophilic due to their short carbon chains between oxygen bridges and POP center is hydrophobic due to larger propylene unit. With this amphiphilic nature, this non-ionic surfactant can readily penetrate itself into the plasma membrane, form a patch preventing the

leakage of the intracellular components and help maintaining ionic concentration across the cell bilayer membrane (Maskarinec et al., 2002). The ability of the surfactants to repair damaged cell membranes due to mechanical trauma or toxic chemical exposure of the cells (i.e. neuronal cells, cartilage cells) has been documented by several groups (Lee et al., 1999, Hellung-Ransel et al., 2000, Marks et al., 2001, Phillips et al., 2004, Rundell et al., 2005)

Phillips et al. (2004) studied the potential of a non-ionic surfactant P188 (8 mg/ml) of saving traumatized chondrocyte in a chondral explant system. They observed that the percentage of live cells increased 24 h after the administration of the surfactant. As live cells in P188-treated explants were significantly greater than that of P188 untreated group, it was deduced that P188 surfactant could help restore the integrity of cell membranes in cartilage damaged by blunt mechanical trauma. The authors also discussed the point that P188 repaired chondrocytes might die eventually, however, this can be prevented with the addition of a caspase inhibitor.

Rundell and his coworkers (2005) recently studied the effect of early injection of poloxamer 188 (P188) into rabbit patello-femoral joint on the percentage of necrotic cells in traumatized cartilage after a severe impact. They found out that significantly fewer cells (7 %) suffered from necrosis in the poloxamer injected group, most markedly in the superficial cartilage layer compared to no poloxamer injected ones. Therefore, it was concluded that the use of P188 surfactant early after a severe trauma to articular cartilage might allow sufficient time for damaged cells to heal (Rundell et al., 2005).

1.2.2.3. Raloxifene, A Selective Estrogen Receptor Modulator

Raloxifene (Ral) is a selective estrogen receptor modulator (SERM) drug that is used currently for treatment of postmenopausal osteoporosis. However, it is less effective in reducing bone turnover and preventing bone loss than estrogen (Lufkin et al., 1998, Preestwood et al., 2000, Reid et al., 2004). Recently, Rogers et al. (2007) investigated the different effects of these two agents on the production of the pro-resorptive cytokine IL-1β and its IL-1 receptor antagonist (IL-1ra) with *in vitro* (whole blood cell culture) and *ex vivo* studies (LPS-stimulated whole blood cell culture). Their work showed that treatment with Ral unlike estrogen did not modulate the production of the proinflammatory cytokines IL-1β and IL-1ra and conclude that this might account for the reduced efficacy of the drug on bone mineral density turnover. On the other hand, Allen and his coworkers (2006) showed that even though Ral treatment does not involve increase of bone mineral density, it produces improvements in bone mechanical properties like strength, stiffness and energy to fracture.

Researches and clinical uses of Ral have showed other beneficial effects, namely a significant (72 %) decrease in new cases of breast cancer and a significant reduction in the incidence of cardiovascular events in women with increased cardiovascular risk (Vogel et al., 2006). This drug acts as an estrogen agonist on bone density and an estrogen antagonist on breast and uterine tissue (Akesson, 2003). Nilsson et al. (2003) also showed that Ral, an estrogen agonist, accelerates growth plate senescence and hastens the epiphyseal fusion.

As a second beneficial potential, inhibitory effect of the drug on cartilage degradation was suggested by some other researchers. In order to show this effect a procedure was followed to assess the effect of ovariectomy on cartilage turnover and degradation. It was concluded that both estrogen and the selective estrogen receptor modulator inhibited the ovariectomy-induced acceleration of

cartilage and bone turnover and significantly suppressed cartilage degradation and erosion in ovariectomized rats. This indicated that the selective estrogen receptor modulator in addition to estrogen plays an important role in cartilage turnover and cartilage surface erosion (Høegh-Andersen, 2004).

Another study investigating the role of SERM group in treatment of cartilage degradation came from Christgau et al. (2004). The preventive role of estrogen and levormeloxifene, another SERM on the ovariectomy-induced changes in cartilage degradation was investigated by this group. It was demonstrated that SERM suppressed cartilage degradation in both rodents and humans, suggesting potential therapeutic benefits in the prevention of OA (Christgau et al., 2004). Also, the clinical trials of 12 month-Ral administration on women suffering from knee OA revealed its anti-osteoarthritic effect (Daniluk et al., 2005, Badurski et al., 2005).

A recent work by Bellosta and his coworkers (2007) showed that Ral reduced the activity of MMP 9 in cultured macrophages and smooth muscle cells. In addition to the *in vitro* data they showed that Ral treatment of ovariectomized rabbits fed with 1 % cholesterol rich diet resulted with reduced expression and activity of MMP 2, 3 and 9 and lower intimal thickening. This second generation SERM treatment might have benefits and great potential in

treatment of many pathological conditions of tissue destruction seen in cancer invasion, cartilage destruction in arthritis, pulmonary fibrosis, neuroinflammation, etc. Additionally, the potent effects in its enhancement of antioxidant defense system by increasing antioxidant enzyme activities and thereby reducing the lipid peroxidation was also recently shown by Ozgocmen and his coworkers (2007).

1.3. Aim of Study

OA is an articular cartilage disease characterized by the progressive degradation and loss of cartilage. There is no effective treatment for the disease. The currently used strategies are mainly towards relieving symptoms. In this study, it was aimed to study the therapeutic potential effects of BMP-9, Ral and PLF-68 on OA with a three-dimensional Aza-C-induced *in vitro* model. The *in vitro* OA model was established by embedding rat articular chondrocytes into 4 % agarose to obtain the agarose-chondrocyte discs, cultivating for 10 days in chondrocyte differentiation medium under hydrostatic pressure (maximum 0.2 MPa at a frequency of 0.1 Hz at 32°C for 30 min. daily) and exposing to Aza-C for 48 hours at day 10. In order to investigate the effectiveness of BMP-9, Ral and PLF-68 on the treatment of osteoarthritic chondrocytes, the effects of different doses of these agents on cell number, matrix synthesis (GAG and collagen) and mechanical properties of the agarose-chondrocyte discs were tested after 10 days of treatment period using MTT viability assay, quantitative colorimetric assay, histochemical staining and stress-relaxation tests, respectively. Healthy articular chondrocytes and untreated Aza-C-exposed articular chondrocytes (control) embedded in 2 % agarose discs were used for comparison of the efficacy of BMP-9, Ral and PLF-68.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM)/HAM'S F-12, DMEM (high glucose), DMEM (low glucose), trypsin (0.25 %), collagenase type II, fetal calf serum (FCS), non-essential amino acids (MEM), streptomycin, sodium pyruvate solution, trypsin-EDTA and bovine serum albumin (pH 7.0) were purchased from PAA Laboratories GmbH (Austria). L-ascorbic acid (99 %), glycine, 1,9 dimethyl methylene blue (DMMB) were obtained from Aldrich (Germany). Agarose type VII, trypan blue solution (0.4 %), human transforming growth factor-β1 (TGF-β1), L-proline (non-animal source), bicinchoninic acid solution, cupric sulfate pentahydrate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (99 %) and dexamethasone (97 %), insulin-transferrin-sodium selenite (ITS) liquid media supplement, chondroitin sulfate from bovine trachea Type A, papain, Raloxifene (Ral), 5-Azacytidine (Aza-C) and Pluronic F-68 (PLF-68) were the products of Sigma Chemical Corporation (USA). L-cysteine, di-sodium hydrogen phosphate dihydrate and di-potassium hydrogen phosphate anhydrous, safranin T, Direct red 80 (Sirius red), Alizarin red S and calcium carbonate were purchased from Fluka Chemical GmbH (Switzerland). Sodium chloride was obtained from Riedel-de Haën (Germany). Dimethyl sulfoxide (DMSO) (molecular biology grade) was supplied by AppliChem GmbH (Ger-

many). Methylthiazolyldiphenyl-tetrazolium (MTT) bromide thiazolyl blue bromide was purchased from GERBU Biochemicals GmbH (Germany). 96 % ethanol was obtained from Ryssen (France). Hank's salt solution (10X) and cetylpyridinium chloride were supplied by Biochrom KG (Germany). Bone Morphogenetic Protein-9 (BMP-9) was purchased from Abnova Corporation (Taiwan).

2.2. Methods

2.2.1. Articular Chondrocyte Culture

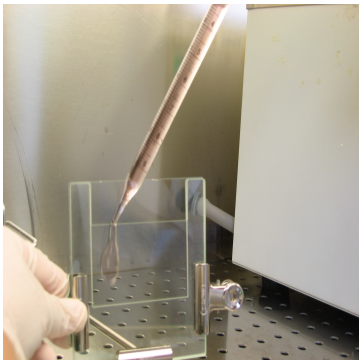
2.2.1.1. Isolation of Articular Chondrocytes from Newborn Sprague-Dawley Rats

Cartilage was taken from the joints of hind leg knees of 6-day-old Sprague-Dawley rats. The skin and soft tissues were removed and the femurs of the hind legs were dislocated. The soft tissues surrounding the joints were scraped off and the articular cartilage were cut off. The cartilage pieces were treated with 10 ml of 0.1 % trypsin/g cartilage for 45 min at 37°C with shaking to remove remaining soft tissues. They were then subjected to 100U/ml collagenase type II in Hank's Salt for 10.5 h. The cell suspension was diluted with Hank's Salt in a 1:3 ratio and filtered through a sterile 100 µm cell strainer. The filterate was centrifuged at 1400 rpm for 10 min in a centrifuge (Hettich Zentrifugen, EBA 20, Germany). Chondrocytes obtained were cultured in DMEM/HAM'S F-12 medium supplemented with 10 % FCS, 50 µg/ml ascorbic acid, 0.1 mM non-essential aminoacid, 0.4 mM proline and 0.1 % streptomycin at 37°C under 5 % CO_2 in a carbon dioxide incubator (SL SHEL LAB, Faster, Italy) (Salva et.al, 2005). Supernatant was diluted and then centrifuged in the same way mentioned above. This procedure was repeated two more times. The growth medium was changed every third day and the chondrocytes were passaged with

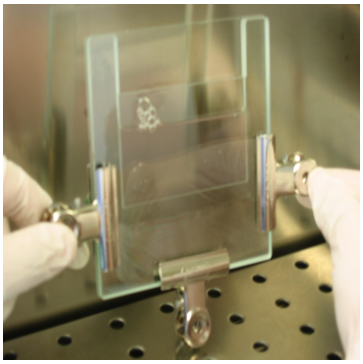
0.1 % trypsin-EDTA solution in a 1:3 ratio. The photographs of cells were taken at certain time points using an inverted microscope (Nikon ECLIPSE, TS 100/TS 100-F, Japan). Chondrocytes at 3^{rd}-4^{th} passage were used for all cell culture studies.

2.2.2. Three-Dimensional Construction of Articular Chondrocyte Culture and Development of *In Vitro* OA Model

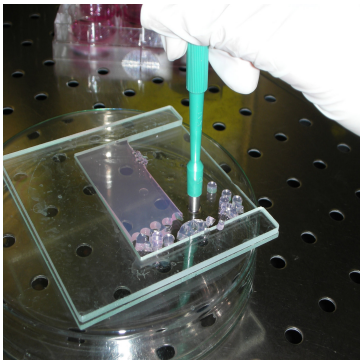
In order to construct a three-dimensional (3-D) *in vitro* chondrocyte culture, mimicking the natural environment of these cells agarose-chondrocyte discs were prepared. Briefly, 4 % (w/v) agarose in phosphate buffered saline (PBS) (0.01 M, pH 7.2) was prepared and sterilized with autoclaving (ALP CL-40M, Labomar, Turkey) at 121^{o}C for 20 min. Chondrocytes were detached from the tissue culture flasks by incubating in 0.1 % trypsin-EDTA for 5 min at 37 °C. The cell suspension was centrifuged for 5 min at 2000 rpm, and the pellet was resuspended in differentiation medium consisting of DMEM/HAM'S F-12 supplemented with 2 % FCS, 1 % ITS , 1 % sodium pyruvate, 100 nM dexamethasone, 80 μM ascorbic acid and 0.1 % streptomycin. Viable cell counting was done with trypan blue staining. $4x10^6$ cells/ml was mixed with an equal volume of agarose (4 % w/v) at 38°C in a laminar flow cabinet and cast between slab gel electrophoresis plates separated by 3 mm thick glass spacers as shown Figure 2.1. (Buschmann et al, 1995). After gelling at room temperature for 15 minutes, approximately 80 discs, 5 mm in diameter and 3 mm in height were punched using a sterile skin punch. The agarose-chondrocyte discs were cultured on top of 1 mm size stainless steel mesh to promote nutrient diffusion from below and above. Each disc was fed every 3 days with the differentiation medium up to day 10. 10 ng/ml TGF-β1 was also added to the differentiation medium of the agarose discs for 7 days.



a)



b)



c)

Figure 2.1. Stages in the preparation of agarose-chondrocyte discs, a) Pouring agarose-chondrocyte suspension between slab gel electrophoresis plates, b) After casting agarose-chondrocyte suspension, c) Punching the agarose discs using a sterile skin punch.

The discs were subjected to hydrostatic pressure amplitude of maximum 0.2 MPa at a frequency of 0.1 Hz at 32°C for half an hour in a custom-made pressure chamber (Biolab Ltd, Turkey) 5 days a week both to mimic articular cartilage microenvironment and to improve chondrocyte cell culture conditions (Figure 2.2.).

At 10th day of incubation, 15 µg/ml of Aza-C was added to the medium of agarose discs. After 48 h, Aza-C was removed and the discs were cultured in the same medium without Aza-C for additional 5 days for *in vitro* OA model development (Ho, et al, 2006).

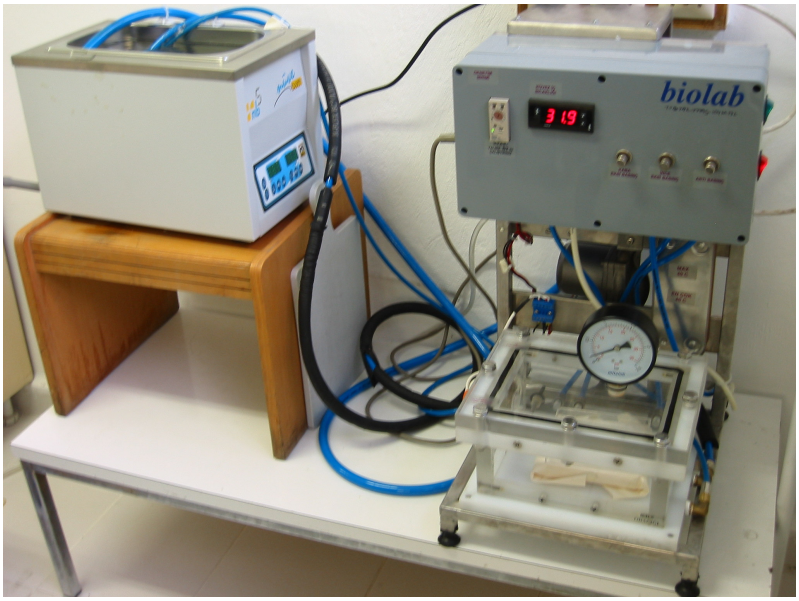


Figure 2.2. Custom-made pressure chamber

2.2.3. Investigation of Therapeutic Effects of BMP-9, Ral and PLF-68 in a 3-D *In Vitro* OA Model

Therapeutic potentials of BMP-9, Ral and PLF-68 were investigated in a 3-D OA *in vitro* model using rat articular chondrocytes. OA *in vitro* model was constructed as described in Section 2.2.2. The media of the agarose-chondrocyte discs were changed every 3 days. The therapeutic agent was added with every medium change.

2.2.3.1. Determination of Dose Effects of BMP-9, Ral and PLF-68 for Treatment of OA

The effects of different doses of BMP-9, Ral and PLF-68 (Table 2.1.) were studied. Aza-C induced osteoarthritic cells embedded in agarose were used for evaluation of therapeutic potential of these agents for OA. The dose-effect relation for each agent on Aza-C induced osteoarthritic cells was determined by evaluating the cell number, total sulfated GAG, collagen and protein contents, wet weights and mechanical properties of the discs for all doses used as described below. Aza-C exposed and untreated articular chondrocytes (control) and healthy articular chondrocytes embedded in agarose were used for comparison.

Table 2.1. Dose regime of the therapeutic agents

Therapeutic Agent	**Dose**		
BMP-9 (ng/ml)	5	10	50
Ral (μM)	1	5	10
PLF-68 (mg/ml)	8		12

2.2.3.1.1. Wet Weight

The wet weights of agarose-chondrocyte discs were obtained after 27 days of culture. The discs were then frozen in liquid nitrogen and lyophilized in METU Central Research Laboratory for 6 hours for sGAG, collagen and protein analysis.

2.2.3.1.2. Cell Number

Cell number of agarose-chondrocyte discs was determined via MTT assay (Vinardell et al., 2006). MTT in DMEM low glucose medium (5 mg/ml) was added to each well. The discs were incubated for 4 h at 37 °C and 5 % CO_2 in the darkness. MTT was then taken out and the wells were washed with PBS. Lastly, DMSO was added to the wells and the plate was incubated for 10 minutes with shaking to solubilize insoluble formazan crystals inside the chondrocytes. Absorbance at 550 nm was read by a microplate spectrophotometer (BioTek μQuant, USA). The calibration curve for which the standards were prepared from chondrocytes embedded in agarose discs (5 mm in diameter, 3 mm in height) was used to quantify the cell number per disc (Appendix A).

2.2.3.1.3. Glycosaminoglycan Content

The sulfated glycosaminoglycan (sGAG) content of agarose-chondrocyte discs and media collected during 10 days treatment was determined by using dimethylmethylene blue (DMMB) assay (Farndale et al., 1986). 16 μg DMMB/ml (pH 3) was prepared in Glycine/NaCl solution containing 3.04 g Glycine, 2.37 g NaCl, 95 mL 0.1 M HCl and 905 mL distilled water. Chondroitin sulfate (ChS) from shark cartilage was used as a standard. A calibration curve was constructed using different amounts of ChS standard (0-5 μg) in Phosphate Buffered EDTA (PBE) solution (containing 14.44 mM cysteine,100 mM di-sodium hydrogen phosphate,10 mM EDTA, pH 6.5).

For determination of total sGAG, the agarose discs and media collected were digested with papain type III (125 μg/ml containing 2 mM dithiothreitol) at 60°C for 20 h. After digestion, discs were incubated at 70°C for 10 min to melt agarose. The samples were vortexed and sedimented at 12000 rpm for 5 min

with a centrifuge (Eppendorf 5804 R, Germany) at 10˚C. 50 µL of the supernatant was mixed with 1.25 mL DMMB dye solution in a 24-well plate and the absorbance at 525 nm was determined immediately with a microplate spectrophotometer (BioTek µQuant, USA). sGAG content of the discs were quantified by the corresponding calibration curve (Appendix B).

2.2.3.1.4. Collagen Content

A protein binding assay was used for measurements of collagen secretion using Sirius red S (100 µg/ml). Sirius red S binds specifically to the [GLY-X-Y]n helical structure found in all collagen types. Briefly, 200 µl aliquot from papain digested agarose was dried in 37˚C dry oven for 48 hours. The dye then was added for 1 hour incubation at room temperature on an orbital shaker (BIOSAN, OS-10, Turkey). After Sirius red S was removed, the samples were extracted with 0.1 N NaOH for 30 minutes and the absorbances were measured at 550 nm using a microplate reader (BioTek µQuant, USA). Bovine collagen from tracheal cartilage was used as a standard for construction of the calibration curve (Appendix C) (Na et al., 2006). The calibration curve constructed was used for quantitation of collagen per disc and percent of wet weight.

2.2.3.1.5. Protein Content

Bicinchoninic acid (BCA) assay was used to determine the protein content of the agarose discs. Bovine serum albumin was used as a standard protein in the construction of a calibration curve (Appendix D). 50 µl of each standard solution and papain digested agarose sample were mixed with 1 ml cupric sulfate-BCA reagent and were incubated at 37 °C for 30 min. At the end of 30 minute-incubation, absorbances at 562 nm were read with a microplate spectrophotometer (BioTek µQuant, USA) (National Institute of Health

Sciences, 2006). The protein content of the agarose discs were determined using the calibration curve.

2.2.3.1.6. Histological Analysis

Agarose discs were frozen in liquid nitrogen and cryosections of 10 μm thickness were taken using a cryostat (Leica). The cryosections were mounted on SuperFrost microscope slides. The sections were fixed with 100 % alcohol and stained with Safranin O for sGAG analysis. The cryosections for collagen analysis were fixed with 4 % formaldehyde solution for 10 minutes. The Sirius red S was used to examine collagen distribution. Images were taken from the stained sections for histological evaluation.

2.2.3.1.7. Mechanical Properties

A computer-controlled testing device (Lloyd LS 500, UK) equipped with Nexygen MT Software Version 4.5 (Ametek Inc., UK) and a 10 N load cell was used to perform unconfined compression tests on agarose-chondrocyte discs. All tests were carried out between two rigid-impermeable platens at room temperature. Before each test, free swelling disc thickness and diameter were measured via a digital caliper. Discs in PBS were first subjected to unconfined stress-relaxation test with a ramp speed of 3 mm/min until reaching 10 % of the free swelling thickness of the discs. Tests were completed after the discs relaxed to equilibrium (600s). The equilibrium unconfined compression (Young's) modulus (E_Y) (equilibrium aggregate modulus) was calculated from the equilibrium stress divided by applied strain.

Figure 2.3. Set up used in stress-relation tests of agarose-chondrocyte discs.

2.2.4. Statistical Analysis

Data were analyzed using One-Way ANOVA and Tukey's Multiple Comparison Tests for the post-hoc pairwise comparisons using SPSS-9 Software (SPSS Inc., USA). Differences were considered significant at $p \leq 0.05$ level. However, the differences at $p \leq 0.1$ level were also shown at certain specific situations.

CHAPTER 3

RESULTS AND DISCUSSION

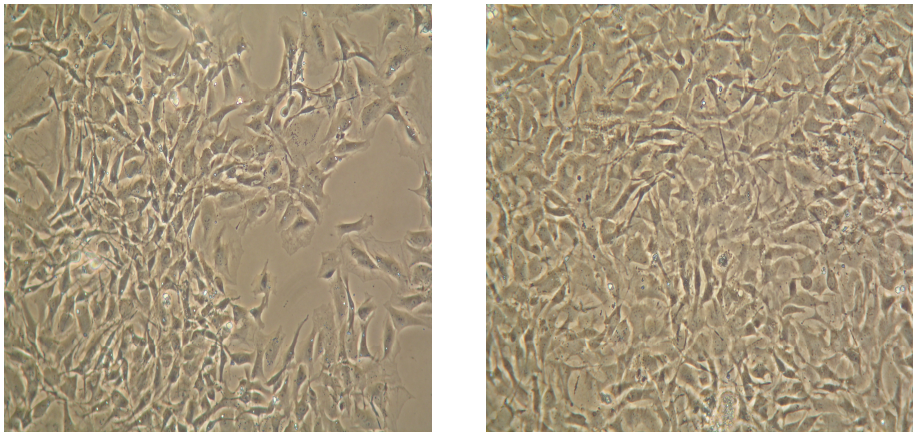
3.1. Articular Chondrocyte Culture

Chondrocytes were isolated from articular cartilage of newborn rats with sequential trypsin and collagenase digestion. The cells had the typical chondrocyte morphology as seen in Figure 3.1.a in accordance with literature.

The rat articular chondrocytes were passaged up to 4th passage in TCPS before embedding into the agarose gels. Primary rat articular chondrocytes were polygonal in shape (Figure 3.1.a). It was observed that with each passage the cells have lost their characteristic morphology and gained a more fibroblastic appearance (Figure 3.1.b,c). On the other hand, redifferentiation of the chondrocytes was achieved by embedding them into the agarose gel having 3-D shape. The chondrocytes were homogeneously dispersed throughout the agarose in most cases. However, in some places, chondrocytes were also observed in clusters (Figure 3.2).

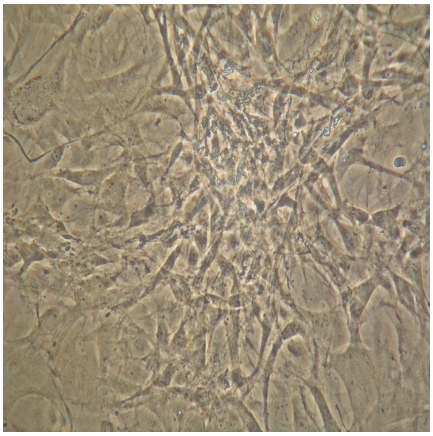
Hydrostatic pressure is a significant component of the mechanical environment in the articular cartilage. Physiological levels of hydrostatic pressure that cartilage experiences is within the range 0.1-20 MPa (Hall et al., 1996). Hydrostatic pressure was applied to the agarose-chondrocyte discs in the custom-made pressure chamber. The dynamic oscillatory mechanical stimulation (5 days of the week with 30 min/day, 0.1 Hz of frequency, maximum 0.2 MPa) used in this study is known to have a positive impact on

3-D chondrocyte culture both by mimicking the natural microenvironment of cartilage and increasing the fluid exchange in the gels. Such increase in the fluid exchange of the agarose discs results with increased access of all the cells embedded to nutrients and differentiation factors. Consequently, redifferentiation of chondrocytes were induced also by cultivation of them in a dynamic 3-D environment within agarose that supported a spherical morphology (Figure 3.2.). Moreover, addition of TGF-β1, ascorbic acid, dexamethasone (glucocorticoid), ITS and proline for 10 days before *in vitro* OA model establishment provided the redifferentiation of the chondrocytes.



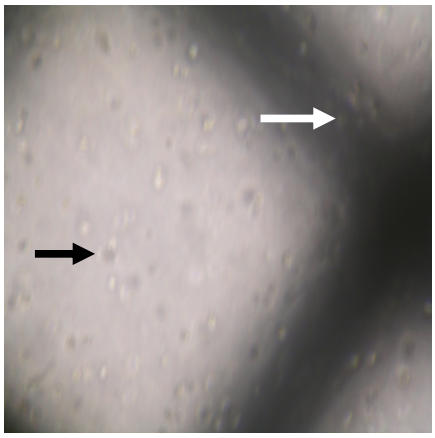
a) b)

Figure 3.1. Phase contrast micrographs of a) primary culture, b)1st passage rat articular chondrocytes, 20X.

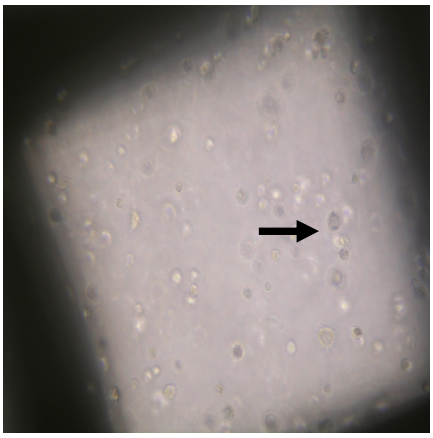


c)

Figure 3.1. (cont'd) Phase contrast micrographs of c) 4th passage rat articular chondrocytes, 20X.



a)



b)

Figure 3.2. Phase contrast micrographs of agarose-chondrocyte discs a) at day 0, b) after 12 days of *in vitro* cultivation (20X). Black arrow points to the group of chondrocytes. White arrow is the mesh underneath the agarose discs.

Additionally, regional opaqueness was observed in the agarose-chondrocyte discs (Figure 3.3., seen as white spots) indicating matrix deposition of the cells into the agarose gels. The white depositions within the gels were observed more at the outer vicinity of the discs.

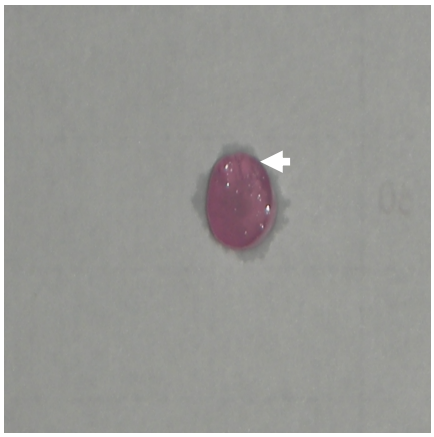


Figure 3.3. The agarose-chondrocyte disc after 27 days of *in vitro* cultivation. White arrow points to the matrix deposition in the agarose disc.

3.2. Investigation of the Effects of Different Doses of Ral, BMP-9 and PLF-68 on Osteoarthritic Cells

Articular chondrocytes embedded in the agarose gels were exposed to Aza-C (15 μg/ml) after 10 days of *in vitro* cultivation. For *in vitro* OA model establishment, the *in vitro* OA model of Ho and his coworkers (2006) was modified. Agarose discs were used instead of alginate beads for embedding chondrocytes. Daily hydrostatic pressure (5 days of the week) was applied to the agarose discs.

3.2.1. Physical Properties of Agarose-Chondrocyte Discs

The agarose-chondrocyte discs were 5 mm in diameter and 3 mm in height at day 0. The dimensions of agarose discs after 27 days of treatment are given in Table 3.1 for all groups. At the end of treatments it was found that there was no statistically significant difference in both dimensions at $p<0.05$ level of significance among the groups.

Table 3.1. Dimensions of the agarose-chondrocyte discs after 27 days of *in vitro* incubation.

	Height (mm)	**Diameter (mm)**
Healthy group	2.78 ± 0.10	4.86 ± 0.04
Control group	3.04 ± 0.20	4.55 ± 0.58
BMP-9 (5 ng/ml)	3.04 ± 0.21	4.83 ± 0.12
BMP-9 (10 ng/ml)	2.85 ± 0.02	4.78 ± 0.07
BMP-9 (50 ng/ml)	2.88 ± 0.06	4.83 ± 0.03
Ral (1 µM)	2.90 ± 0.06	4.60 ± 0.64
Ral (5 µM)	3.02 ± 0.24	4.83 ± 0.17
Ral (10 µM)	2.90 ± 0.16	4.64 ± 0.38
PLF-68 (8 mg/ml)	2.91 ± 0.10	4.99 ± 0.12
PLF-68 (12 mg/ml)	2.77 ± 0.03	5.07 ± 0.06

The comparison of wet weights of discs, however showed significant differences between groups. At the end of 27 days of incubation, the wet weight of the control group was higher than the wet weight of the healthy group (0.0516 ± 0.0041 versus 0.0447 ± 0.0026, $p<0.05$) (Figure 3.4). Previous results showed that OA cartilage exhibits increased levels of cleaved and denatured type II collagen due to high activity of metalloproteases (Bank et al., 1997, Gebhard et al, 2003). Such disruption of collagen network results with the swelling of the tissue (up to 9 % increase in water content) and decrease in the proteoglycans (Mankin and Thrasher, 1975, Bonassar et al., 1995). This could be interpreted as *in vitro* osteoarthritic-like alterations took place in the agarose-chondrocyte discs after Aza-C exposure. The wet weight results also showed that there was no statistically significant difference between the healthy group and all Ral groups. This might be interpreted as Ral also has similar inhibitory effect on the metalloproteinase expression and activity that was previously observed in the macrophages and smooth muscle cells (Bellosta et al., 2007). However, neither BMP-9 nor PLF-68 groups showed the same effect as Ral on the wet weights. The wet weights of these groups were significantly higher than the wet weight of the healthy group.

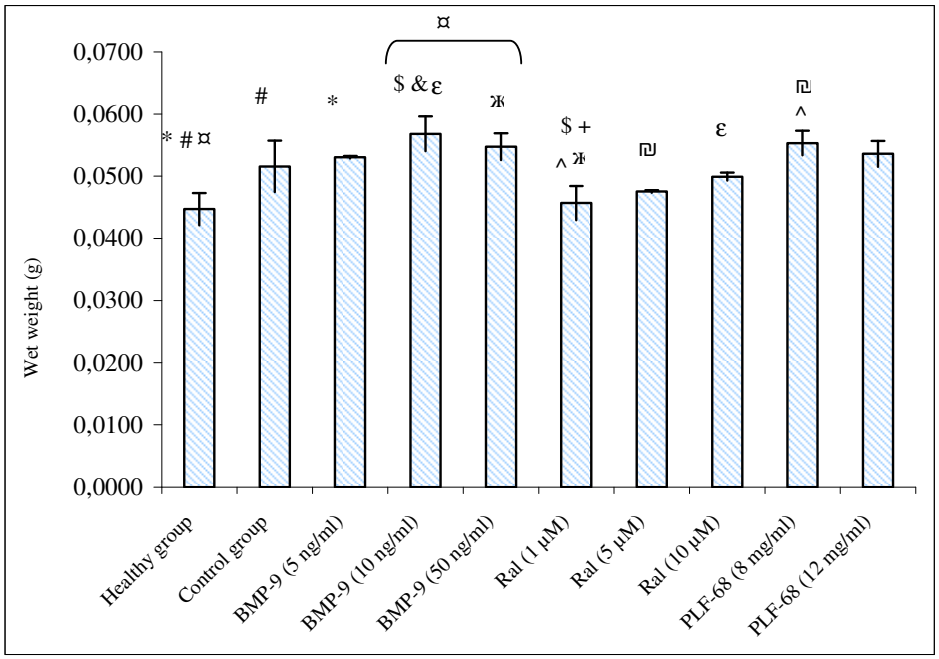


Figure 3.4. Comparison of wet weights of agarose-chondrocyte discs treated with different doses of Ral, BMP-9 and PLF-68 at the end of 27 days of incubation. (Significant differences between groups: #, *, ж, +, &: p≤0.05, $, ^, ¤: p≤0.01, ₪, ε: p≤0.1)

3. 2. 2. Determination of Number of Chondrocytes Embedded in Agarose by MTT Analysis

Initially, 4.00x 10^6 cells/ml was equally mixed with 4 % agarose. The initial cell number in the agarose discs was 200 000 cells/disc. The chondrocytes proliferated in all groups during 27 days of incubation (Figure 3.5). There was no statistical difference between the cell numbers of healthy groups and control groups (untreated Aza-C exposed) (386 990 ± 62 109 vs 421 240 ± 33 757). However, Ho and his coworkers (2006) also used Aza-C treatment on human articular chondrocytes for *in vitro* 3-D OA model development and they observed a 20 % apoptosis in cells embedded in alginate beads using TUNEL staining method.

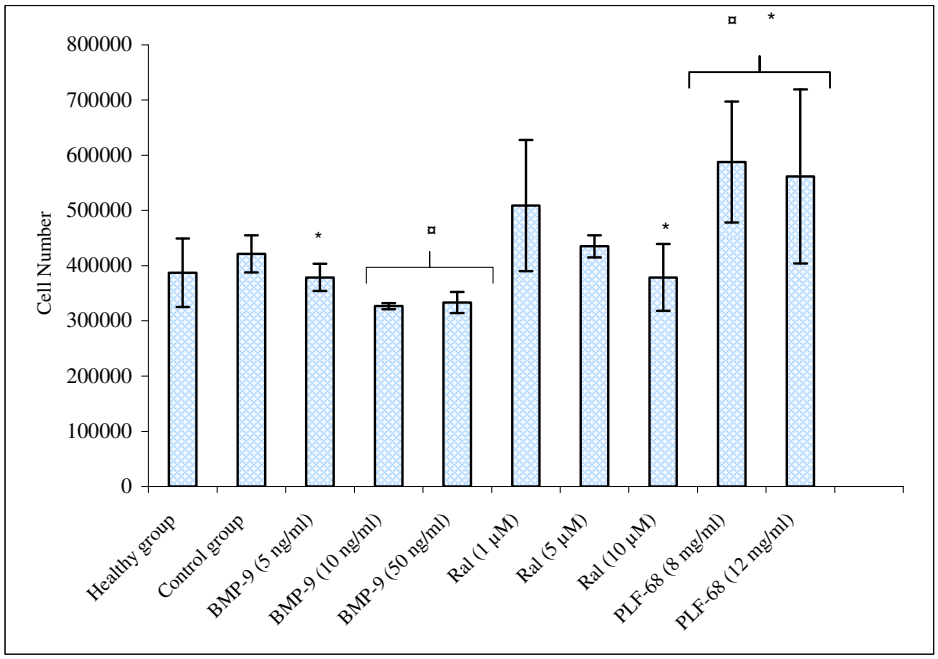


Figure 3.5. The effect of Ral, BMP-9 and PLF-68 treatment on the number of chondrocytes in agarose discs after 27 days of incubation (Significant differences between groups: *: $p \leq 0.1$, ¤: $p \leq 0.05$).

There was a notable increase in the cell number of PLF-68 treated groups (587 490 ± 109 601, 8 mg/ml and 561 657 ±157 824, 12 mg/ml) in comparison to control group (421 240 ± 33 757). This can be interpreted such that the surfactant increased the number of viable cells, despite treatment with Aza-C, by restoring the integrity of cell membranes. Similar effect of surfactants on cartilage during apoptosis was also suggested by Phillips et al (2004). This non-ionic surfactant penetrates itself into the plasma membrane, forms a patch preventing the leakage of the intracellular components and helps to maintain ionic concentration across the cell bilayer membrane (Maskarinec et al., 2002). In the work of Phillips and his coworkers (2004) PLF-68 was shown to restore the cell membrane integrity of mechanically traumatized chondrocytes, rather than the ones exposed to a chemical agent like Aza-C. The effect of PLF-68 on

cell viability in this study was in correlation with the literature on different cell types exposed either to a mechanical trauma or a toxic chemical (Lee et al., 1999, Hellung-Ransel et al., 2000, Marks et al., 2001, Phillips et al. 2004, Rundell et al., 2005).

The cell number of PLF-68 treated group was statistically higher ($p<0.05$) than those of BMP-9 (10 ng/ml) and BMP-9 (50 ng/ml) treated groups. Also the cell numbers of Ral (10 μM) and BMP-9 (5 ng/ml) treated groups were found statistically different from the cell number of PLF-68 treated group at 0.1 significance level ($p=0.084$). This could be due to the settlement of PLF-68 into the membrane rescuing the cells from membrane rupture and eventual death.

3.2.3. Evaluation of Ral, BMP-9 and PLF-68 Treatment on sGAG Synthesized by Osteoarthritic Cells Embedded in Agarose

According to the results of sGAG analysis, Ral (5 μM) treated discs were found to have the highest amount of total sGAG (cumulative amount of sGAG in the disc and released into the medium) (Figure 3.6). The differences between this group and others were found to be statistically significant only for two high doses of BMP-9 ($p\leq0.05$) and Ral (10 μM) ($p\leq0.058$) groups because of large standard deviations within groups and small sample size ($n=3$). The second highest mean value for the total sGAG result was obtained with healthy group. The same BMP-9 and Ral (10 μM) groups also showed significant differences with this group at $p\leq0.05$ and $p\leq0.1$ level of significance, respectively. Both Ral (5 μM) treated and healthy group released significant amounts of their sGAG content into the medium of the chondrocytes after 27 days of incubation in accordance with Mauck et al (2006). The group observed a time dependent sGAG accumulation into the construct and release into the medium after 27 days of incubation.

In general it was observed that the groups had similar trend in sGAG amount per disc. The same significant differences for the total sGAG synthesized by chondrocytes were valid for the sGAG amounts per disc. However, the healthy group had the highest value in this case (Figure 3.6).

The stimulatory effect of BMP-9 on sGAG synthesis was shown in experimental studies using healthy articular chondrocytes (Blunk et al., 2003, Hills et al., 2005). Blunk and his coworkers (2003) used 1, 10 and 100 ng/ml BMP 9 doses in their studies. In comparison to controls, all doses were shown to increase the sGAG synthesis of healthy articular chondrocytes seeded in polyglycolic acid carriers as in the work of Hills et al (2005). They concluded that in contrast to BMP-2, -12, and -13, BMP-9 tended to be active at lower concentrations. However, the amounts of sGAG per disc determined for all BMP-9 dose groups were lower than the amounts of all groups even for the control group in current study. The lower sGAG amount of BMP-9 groups might be due to negative effects of BMP-9 on OA cells despite its stimulatory effects on healthy cells as stated in literature. In a recent work Stöve and his coworkers (2006) treated human osteoarthritic chondrocytes cultured in alginate beads with different doses of BMP-7. The group used the same initial cell number in the constructs as the one in this study. However, they used higher doses of BMP-7 (200, 600 and 1000 ng) than the BMP-9 doses used. The sGAG amount in their study was four fold higher than the sGAG amount determined for all doses of BMP-9 in this study.

The additional significant differences were found for the groups; control versus BMP-9 (10 ng/ml) ($p \leq 0.1$) and PLF-68 (12 mg/ml) versus BMP-9 (two higher doses) ($p \leq 0.05$) or Ral (10 μM) ($p \leq 0.1$).

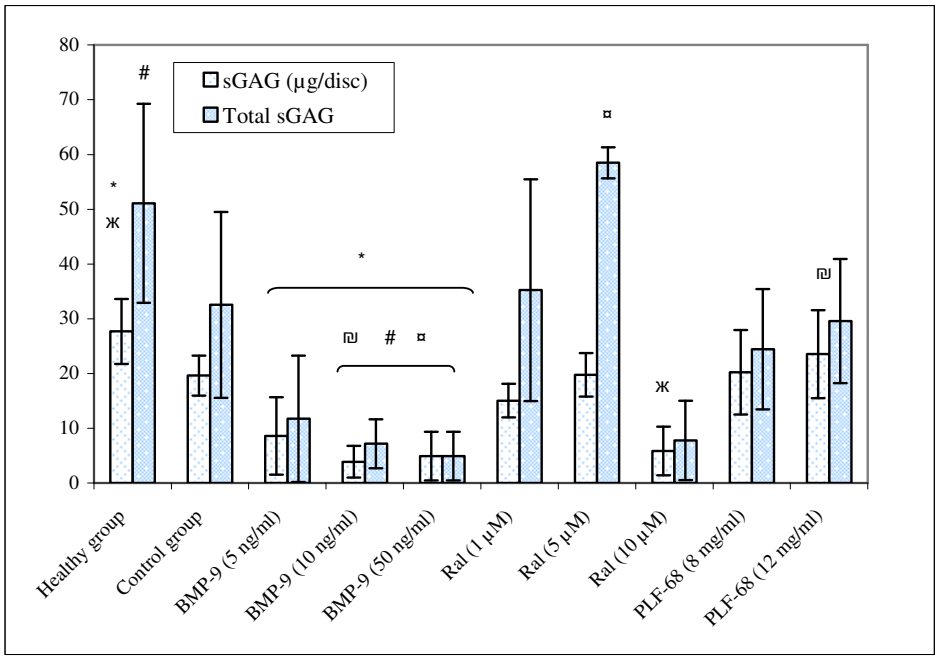


Figure 3.6. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on total sGAG produced by the agarose-chondrocyte discs and the sGAG deposited into the agarose discs at the end of 27 days of incubation. (Significant differences between groups: *, #, ж, ¤, ₪: $p \leq 0.05$)

The comparison of sGAG amounts in terms of percentage of wet weights illustrated more differences between the treatments and control (Figure 3.7). The sGAG content of healthy group was statistically higher than that of the control group ($p<0.1$). The most significant outcome of this comparison was statistically insignificant difference of only Ral (5 µM) treated group with healthy group. This implies that Ral (5 µM) has reversing effect on cartilage damage caused by Aza-C treatment. A decrease in sGAG/ wet weight of the agarose disc was also observed when Ral dose was increased to 10 µM ($p=0.073$). Thus, this indicates that the optimum dose for Ral might be 5 µM

for the current study. Treatments did not show the same positive effect of PLF-68 on the sGAG percent of wet weight as on the cell number in the agarose discs.

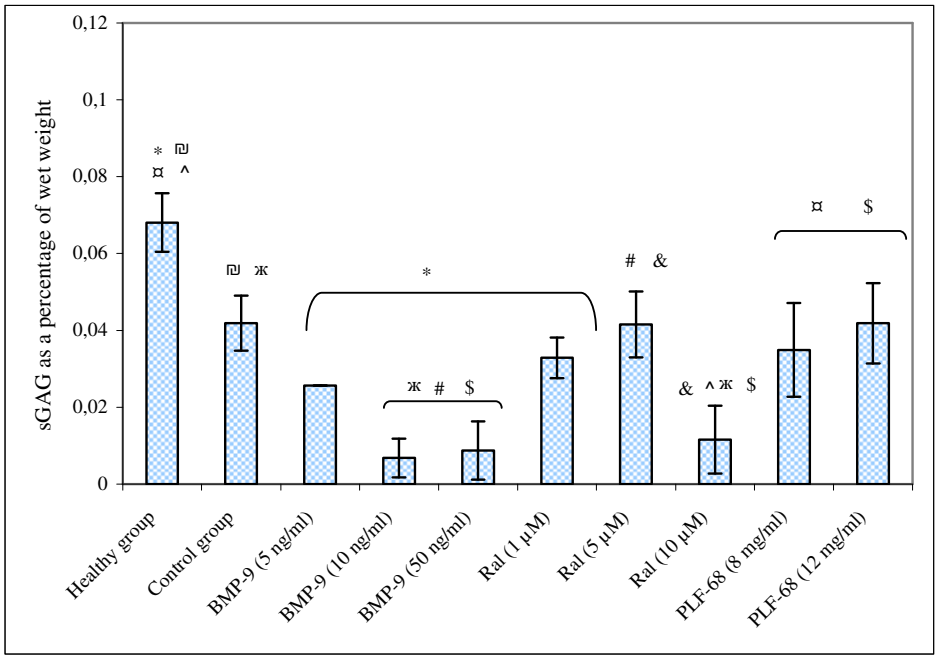


Figure 3.7. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on sGAG percentage of wet weight of the agarose-chondrocyte discs at the end of 27 days of *in vitro* incubation (Significant differences between groups: ₪, &: $p \leq 0.1$ and ^, *, ж, #, ¤, $: $p \leq 0.05$).

3.2.4. Evaluation of Ral, BMP-9 and PLF-68 Treatment on the Collagen Biosynthesis of Osteoarthritic Cells Embedded in Agarose

Collagen is an important extracellular matrix component synthesized by chondrocytes. Collagen plays an important role in the mechanical properties of the cartilage. Previous studies showed that osteoarthritic cartilage *in vivo* can

synthesize elevated amounts of collagen. However, in current study it was observed that although the amount of collagen secreted into the agarose discs in the healthy group was higher than the control group (90.26±11.16 versus 65.91±11.94) in terms of numerical values, the difference was not significant statistically (Figure 3.8). Blunk and his coworkers (2003) observed that BMP-9 lowered the collagen amount synthesized by the healthy chondrocytes. In contrast to the work of Stöve et al, it was observed that 10 ng/ml BMP-9 treatments resulted with an improved synthesis of collagen by the osteoarthritic cells in this study. 12 mg/ml PLF-68 treatment resulted with an increase in the collagen content of the agarose discs which correlated with the increase in cell number observed (Figure 3.5) in comparison to all groups. When the collagen content was normalized to the wet weights of agarose-chondrocyte discs, there was no statistical difference with the healthy group (0.443±0.219 versus 0.197±0.013) owing to its large variation (Figure 3.9). This might imply that PLF-68 might have a stimulatory effect on the collagen synthesis of osteoarthritic cells. A decreasing collagen percent of wet weight was observed as the dose of Ral (1, 5 and 10 μM) increased in correlation with the collagen content/disc results (Figure 3.8).

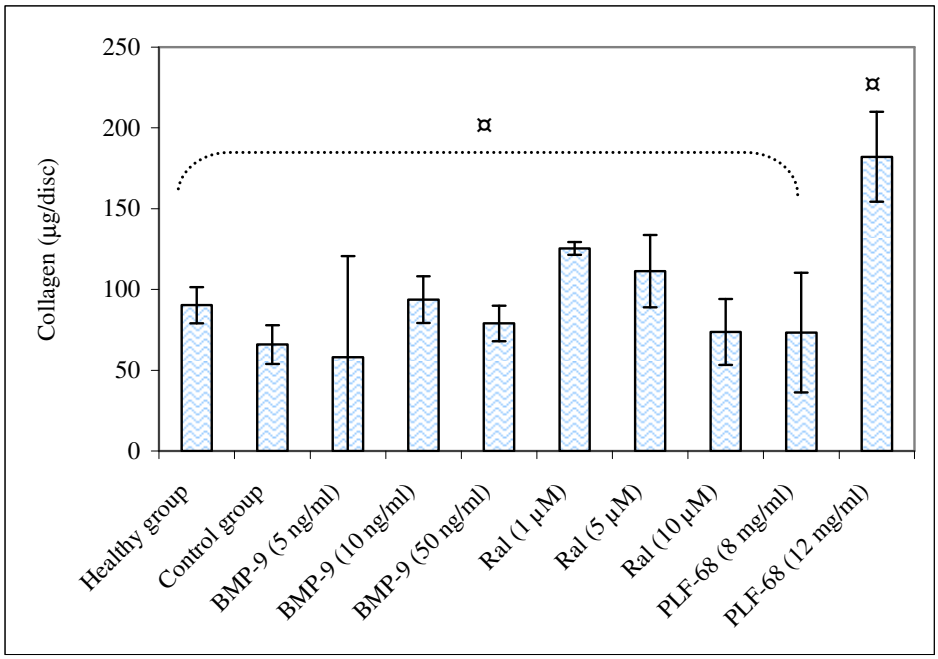


Figure 3.8. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on the collagen content of the agarose-chondrocyte discs at the end of 27 days of *in vitro* of incubation (Significant differences between groups: ¤: $p \leq 0.05$).

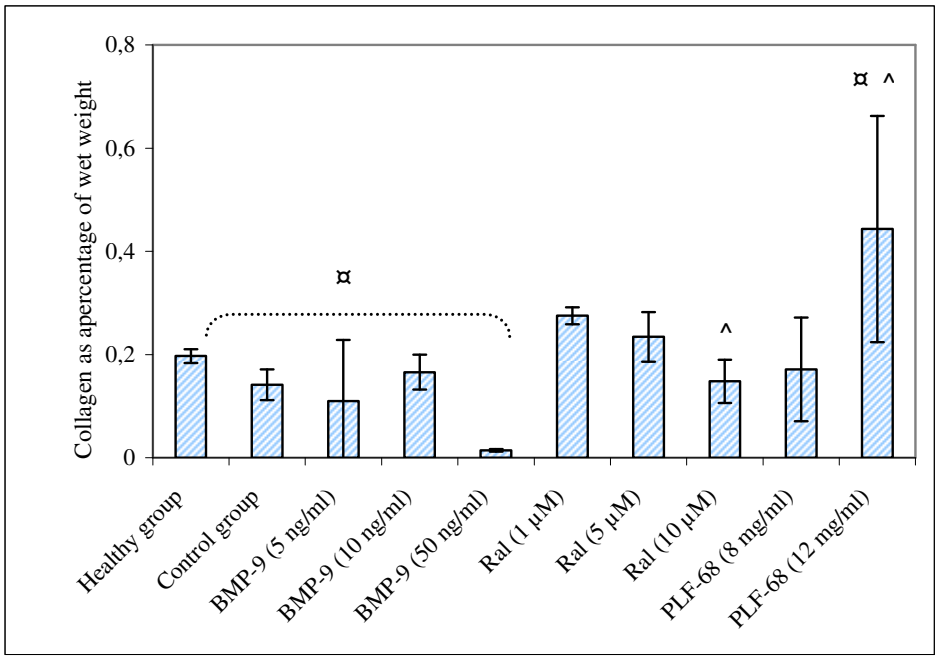


Figure 3.9. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on the collagen percentage of wet weight of the agarose-chondrocyte discs at the end of 27 days of *in vitro* of incubation (Significant differences between groups: ¤, ^: $p \leq 0.05$).

3.2.5. Evaluation of Ral, BMP-9 and PLF-68 Treatment on the Protein Content of Osteoarthritic Cells Embedded in Agarose

The total protein content of the agarose discs were analyzed using quantitative BCA assay. No statistical difference was observed for total protein per disc except for BMP-9 (10 ng/ml) and Ral (1 µM). Additionally, it was obtained that there was no statistical difference for total protein percentage of wet weight among all the groups (Figures 3.10 and 3.11).

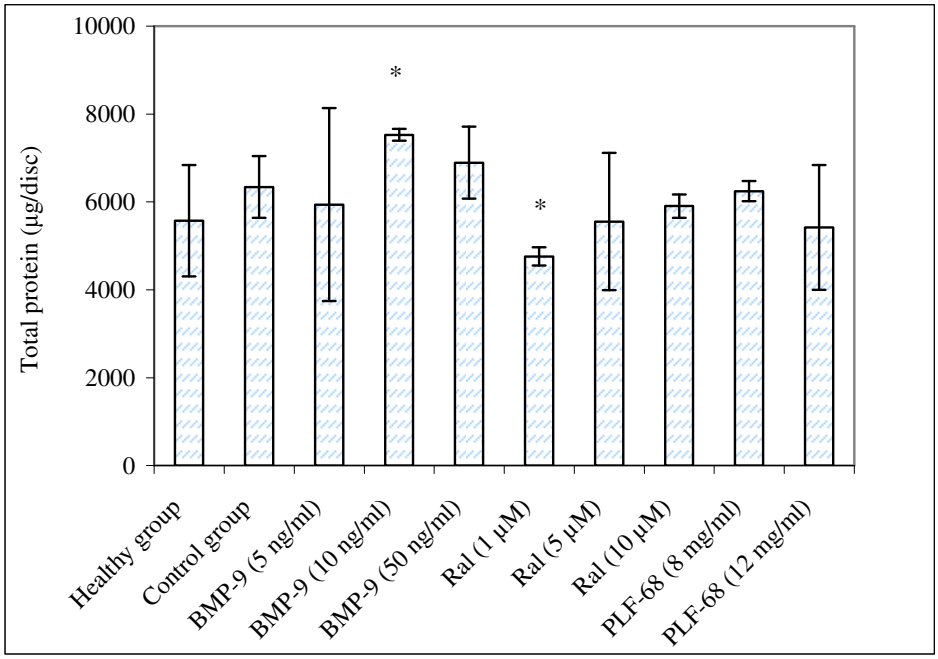


Figure 3.10. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on the protein content of the agarose-chondrocyte discs at the end of 27 days of *in vitro* of incubation (Significant differences between groups: *: $p \leq 0.05$).

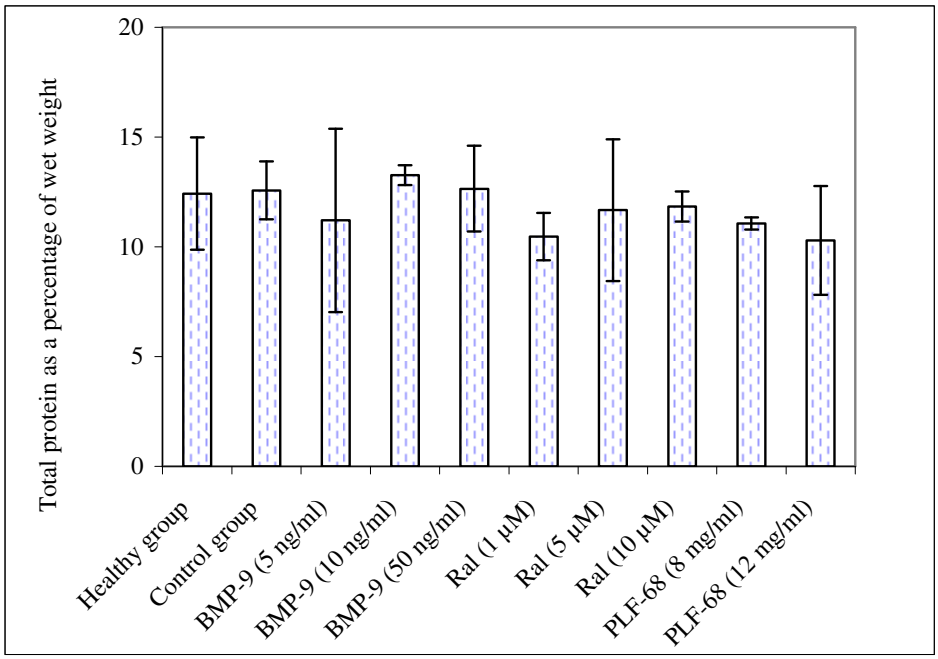


Figure 3.11. Comparison of the effects of different doses of Ral, BMP-9 and PLF-68 on total protein percentage of wet weight of the agarose-chondrocyte discs at the end of 27 days of *in vitro* of incubation.

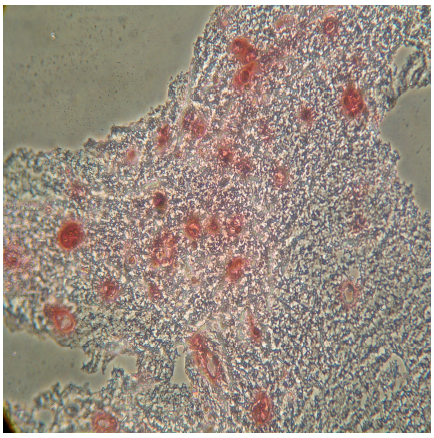
3.2.6. Histological Evaluations

The cells in the cross-sections of gels had round morphology in all groups. The red staining observed in cryosections show the presence of sGAG synthesized by the chondrocytes. Pericellular sGAG staining was present in all groups (Figure 3.12). Matrix deposition of sGAG was observed for healthy group, 5 μM Ral and 5 ng/ml BMP-9 treated groups (Figure 3.12.a, d and f). The healthy group and 5 μM Ral group had the most dense pericellular sGAG staining. Matrix sGAG staining was seen in the regions where there were clusters of cells. However, in the control group only pericellular staining for sGAG was observed (Figure 3.12.b) and the amount of sGAG deposited into

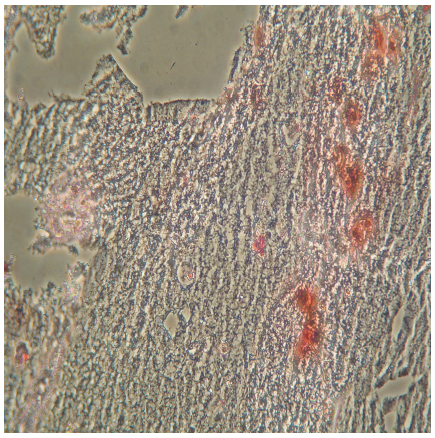
the matrix was very faint. In parallel with the sGAG percentage of wet weight of the disc results (Figure 3.7.), 5 μM Ral group stained heavily for sGAG both in the pericellular and extracellular matrix regions. Ral at this dose therefore can be stated to have a positive effect on the osteoarthritic cells. For 1 μM Ral group, in addition to pericellular staining a faint staining was observed in the matrix region, however, for the highest dose of Ral group such matrix sGAG deposition was not observed (Figure 3.12.c and e).

sGAG staining results for 5 ng/ml BMP-9 was in correlation with the sGAG percentage of wet weight results. The highest amount sGAG percentage of wet weight was observed for this group among the BMP-9 doses used (Figure 3.12. f-h). For the higher two doses of BMP-9 the results were similar to control group.

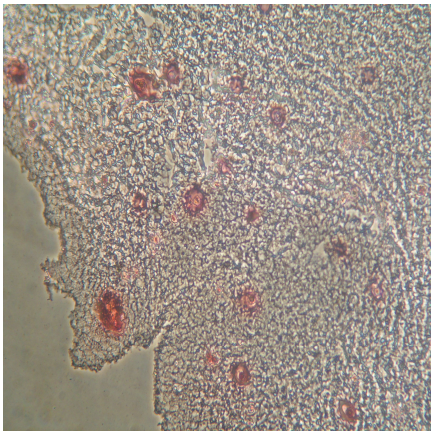
In correlation with the sGAG results in the previous section, PLF-68 treatment did not have a pronounced positive effect on the sGAG synthesis as it had on the cell number. However, dense pericellular and faint matrix stainings were observed for both doses of PLF-68 (Figure 3.12. i-j).



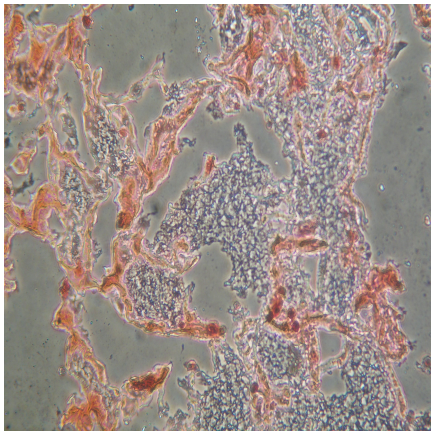
a) Healthy group



b) Control group

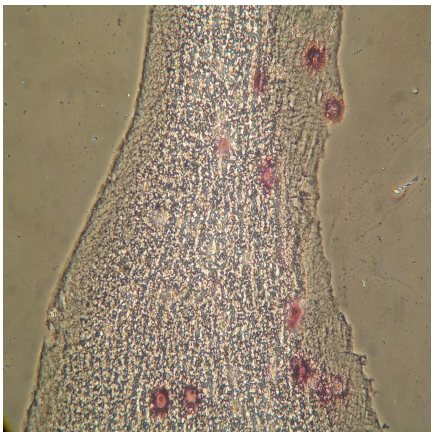


c) 1 μM Ral

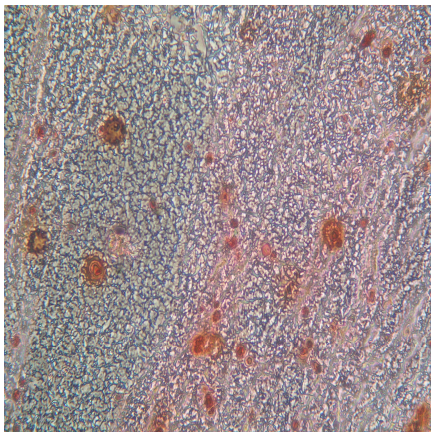


d) 5 μM Ral

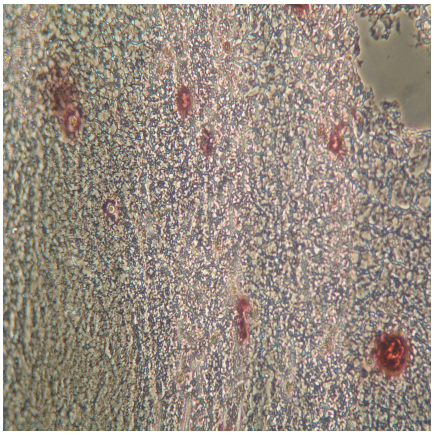
Figure 3.12. The photomicrographs of histology sections of agarose-chondrocyte discs stained with safranin O for sGAG deposited into the agarose discs after 27 days of *in vitro* cultivation.



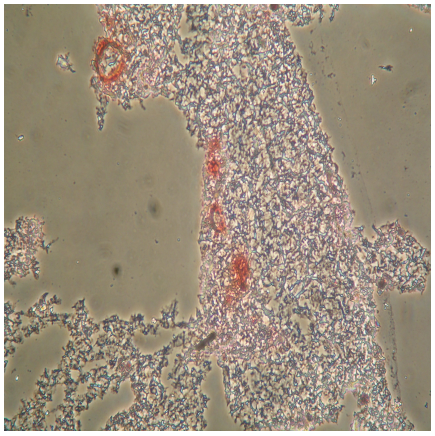
e) 10 μM Ral



f) 5 ng/ml BMP 9

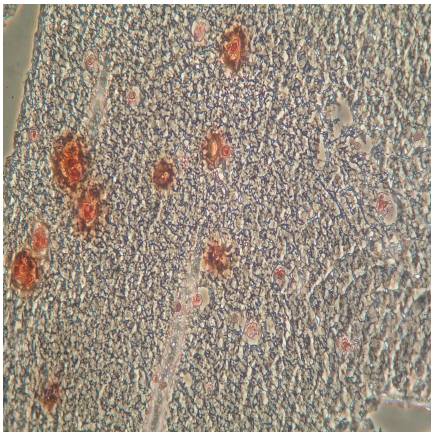


g) 10 ng/ml BMP-9

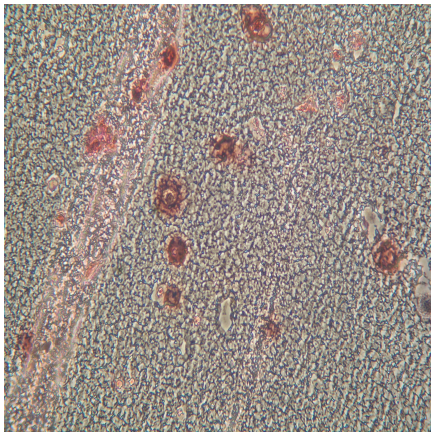


h) 50 ng/ml BMP-9

Figure 3.12. (cont'd) The photomicrographs of histology sections of agarose-chondrocyte discs stained with safranin O for sGAG deposited into the agarose discs after 27 days of *in vitro* cultivation



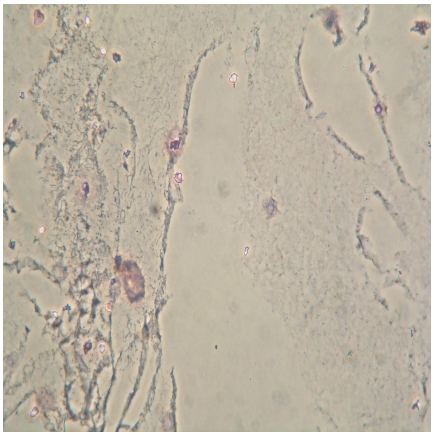
i) 8 mg/ml PLF-68



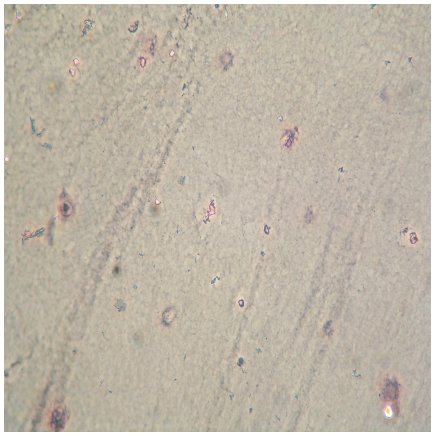
j) 12 mg/ml PLF-68

Figure 3.12. (cont'd) The photomicrographs of histology sections of agarose-chondrocyte discs stained with safranin O for sGAG deposited into the agarose discs after 27 days of *in vitro* cultivation.

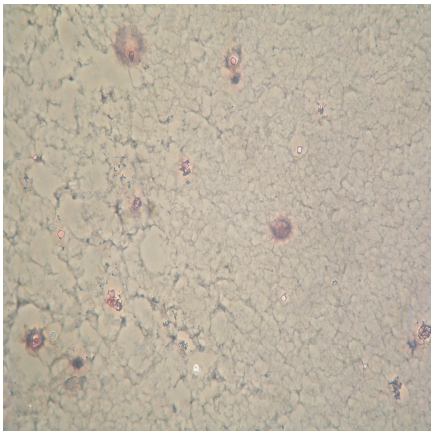
Histologic evaluations of collagen staining showed that only 5 μM Ral treated group had both pericellular and extracellular collagen deposition in the agarose discs among all groups (Figure 3.13). The intensity of staining for this group was remarkably greater than all other groups. In the cryosections of 1 and 10 μM Ral treated groups and 5 ng/ml BMP-9 treated group, a faint staining throughout extracellular matrix was also observed. The other groups were indifferent from each other and had pericellular staining for collagen. From these results it can be concluded that Ral treatment might have a potential in the repair of osteoarthritic cartilage.



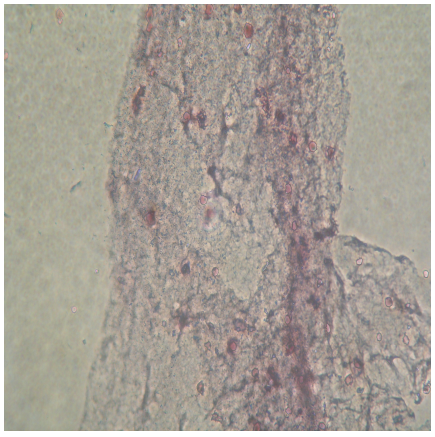
a) Healthy group



b) Control group

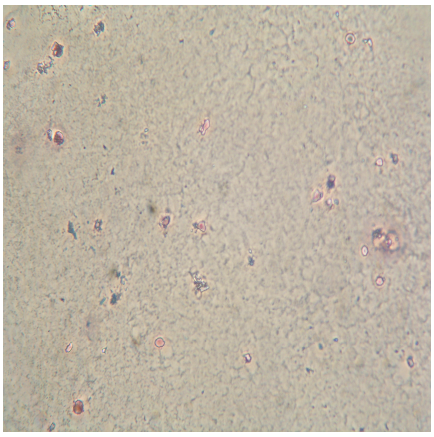


c) 1 μM Ral

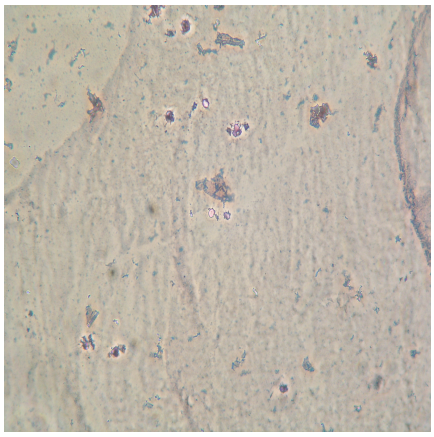


d) 5 μM Ral

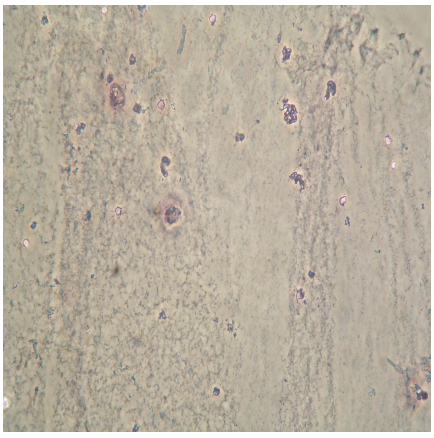
Figure 3.13. The photomicrographs of histology sections of agarose-chondrocyte discs stained with Sirius red S for collagen deposited into the agarose discs after 27 days of *in vitro* cultivation.



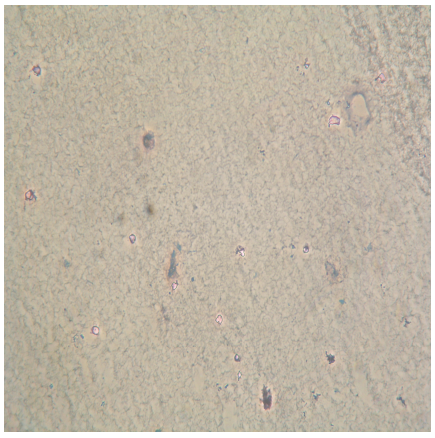
e) 10 μM Ral



f) 5 ng/ml BMP 9

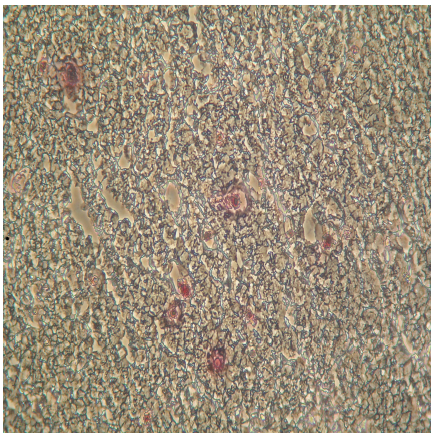


g) 10 ng/ml BMP-9

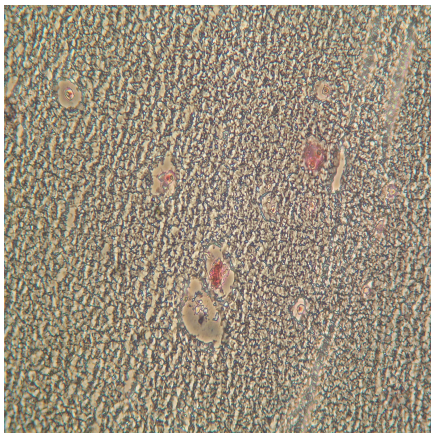


h) 50 ng/ml BMP-9

Figure 3.13 (cont'd) The photomicrographs of histology sections of agarose-chondrocyte discs stained with Sirius red S for collagen deposited into the agarose discs after 27 days of *in vitro* cultivation.



i) 8 mg/ml PLF-68



j) 12 mg/ml PLF-68

Figure 3.13. (cont'd) The photomicrographs of histology sections of agarose-chondrocyte discs stained with Sirius red S for collagen deposited into the agarose discs after 27 days of *in vitro* cultivation.

3.2.7. Comparison of Mechanical Properties of Agarose-Chondrocyte Discs After *In Vitro* Studies

The typical graph of stress-relaxation response of agarose-chondrocyte discs was shown in Figure 3.14. It was observed that stress-relaxation curve observed in all groups were similar in general appearance to theoretical and experimental curves observed in literature for unconfined compression stress-relaxation with slow strain rates (Mauck et al., 2000). This might indicate that the viscoelastic property of the agarose discs was preserved after 27 days of *in vitro* cultivation.

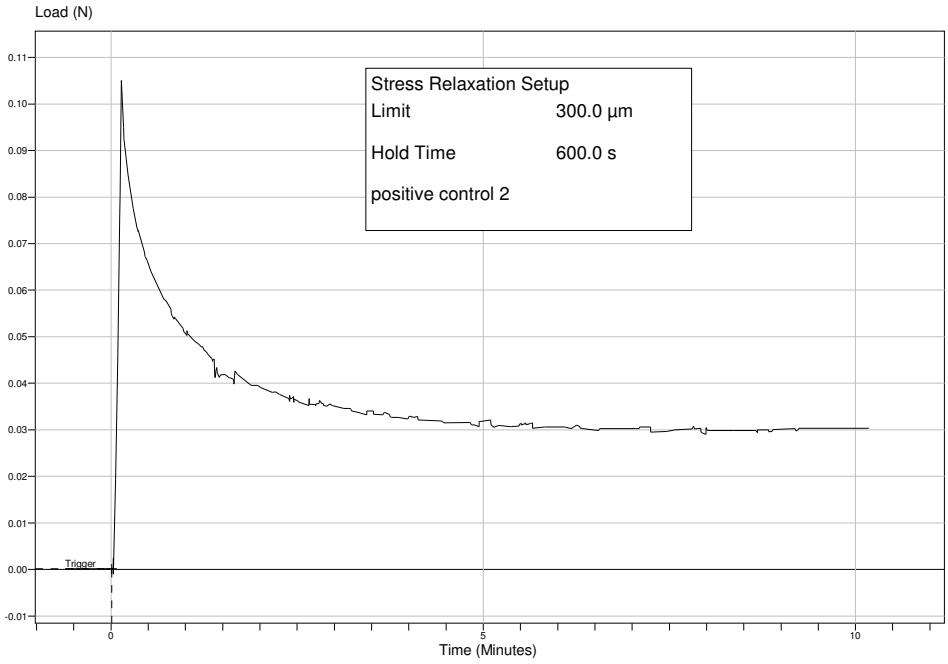


Figure 3.14. Typical unconfined compression stress-relaxation curve during 10 % strain stress-relaxation tests for healthy group of agarose-chondrocyte discs after 27 days of *in vitro* incubation.

From the data of stress-relaxation test of 10 % strain, peak load values were obtained and normalized by the area of each disc to calculate peak stress (Figure 3.15). When compared with healthy group, the peak stress of control group was found significantly different indicating the loss of mechanical properties of agarose-chondrocyte discs upon establishment of OA. Other than control group all BMP groups and two higher doses of Ral were also found to be significantly different than healthy group. Considering the previous results the decline in mechanical properties was an expected outcome for control group and BMP groups which had highest wet weights (Figure 3.4). Also BMP groups and highest dose Ral group's peak stress values were in accordance with their lower sGAG content per disc (Figure 3.6). sGAG is known to be the

main contributers of the compressive strength in cartilage tissue where electrostatic (osmotic) interactions between the proteoglycans of ECM enable the cartilage to resist compressive loads (Buschmann M. D., et al., 1995). In some animal studies the higher proteoglycan content in cartilage of regularly loaded vs immobilized groups were also shown (Buschmann M. D., et al., 1995).

The most important sign of OA is the decrease in the mechanical strength of cartilage matrix formed by chondrocytes. Hence, BMP-9 groups with the lowest peak stress achieved suggest that BMP-9 might be considered to be inefficient in treatment of OA. The increased water content observed could be also suggested as the evidence of functional matrix damage as a result of loss of proteoglycans from the extracellular matrix or due to rupture of collagen fibers in correlation with the literature (Ackermann B., and Steinmeyer J., 2005).

The low dose Ral and both doses of PLF-68, however, had statistically indifferent outcomes in mechanical properties with the healthy group and significantly higher values than both control and BMP groups. This suggests that these agents might have better treatment potential either by inducing chondrocytes towards production of extracellular matrix or by preservation of the integrity of cartilage structure.

The only unexpected outcome of the mechanical tests was the lower peak stress value of Ral (5 μM) group than healthy and Ral (1μM) groups, despite its high sGAG and low wet weight. This might be due to having less number of samples in all groups (n=3), Ral (5 μM) being even lower (n=2).

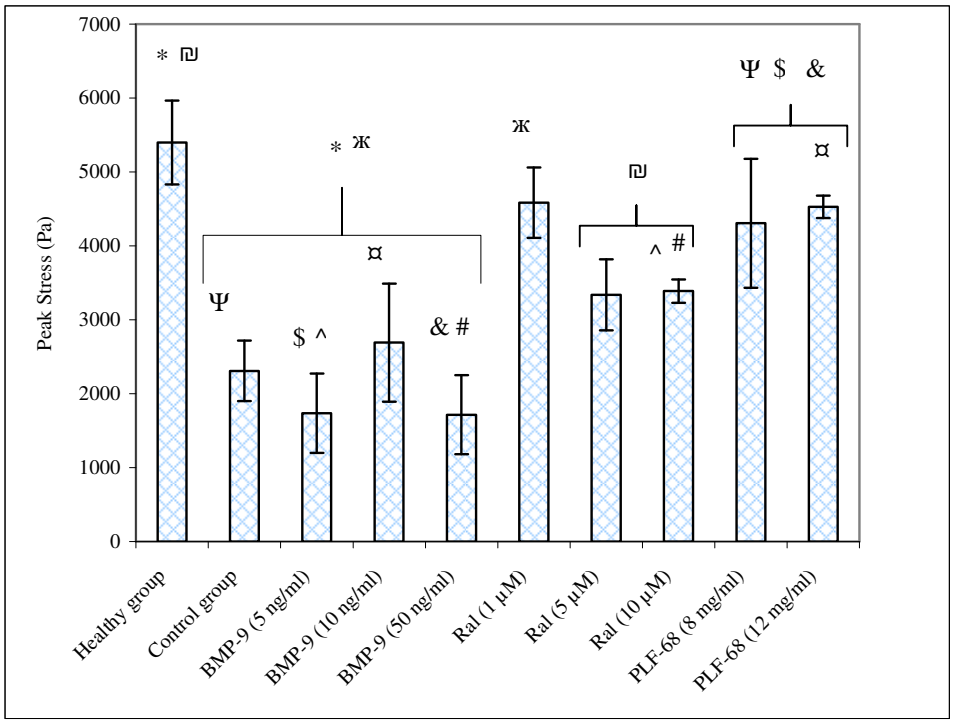


Figure 3.15. Comparison of peak stresses reached during 10 % strain in stress-relaxation tests for treatment, control and healthy groups of agarose-chondrocyte discs after 27 days of *in vitro* cultivation (Significant differences between groups: *: $p \leq 0.002$; ₪, ж, ¤, #, ^, Ψ: $p \leq 0.05$; &: $p \leq 0.02$; $: $p \leq 0.003$, n=3).

The comparison of equilibrium aggregate moduli of groups yielded similar results with peak stress values. The control and BMP 9 groups had significantly lower moduli compared to the healthy group (Figure 3.16). Ral (1 µM) group with the highest equilibrium aggregate modulus among treatment groups was again found to be statistically similar to the healthy group. Also, the difference between the healthy group and other groups of Ral were not significant, showing some degree of treatment effects at these doses. The equilibrium aggregate modulus values of both healthy group and Ral (1 µM)

group (~ 35 kPa and ~ 25 kPa, respectively) were in accordance with that of 2 % agarose-chondrocyte constructs in the literature (~100 kPa), when the cell numbers in the constructs were considered (about 3.5 times more cells were used in that study than the cells in this case) (Mauck R.L., et al., 2000). In the same study it was also shown that the agarose gels with cells have higher equilibrium aggregate modulus values than those without cells.

Buschmann et al (1995) demonstrated that the modulus increased and permeability decreased with time and as matrix was deposited by chondrocytes into 3 % agarose gel.

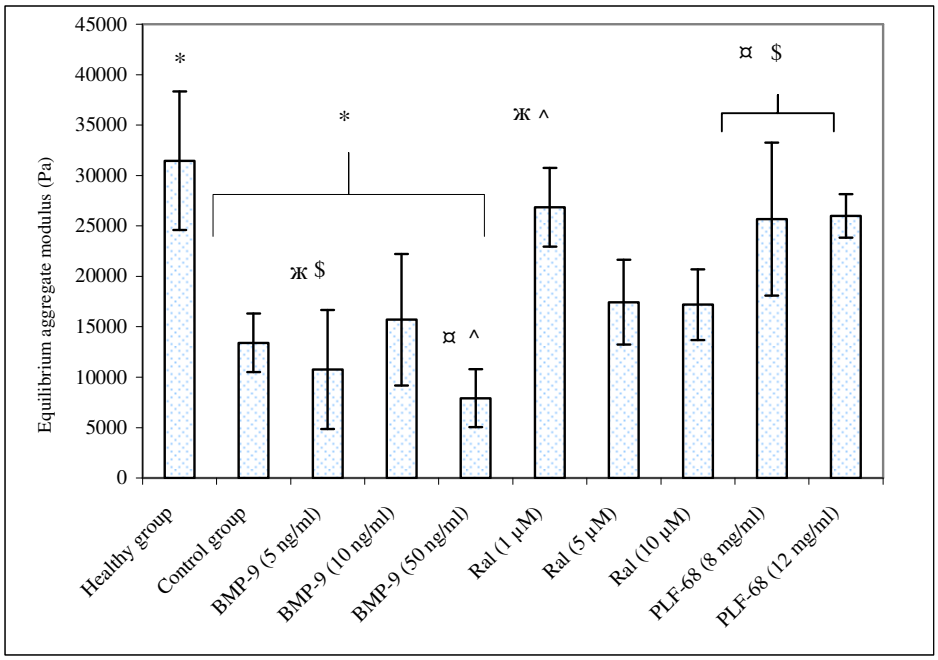


Figure 3.16. Comparison of equilibrium aggregate moduli of agarose-chondrocyte discs for treatment, control and healthy groups after *in vitro* experiments (Significant differences between groups: *, ж: $p \leq 0.05$, ¤, ^: $p \leq 0.02$, $: $p \leq 0.1$).

CHAPTER IV

CONCLUSIONS

OA is one of the world's most incident joint diseases mainly affecting the elder people. There is a growing demand for the development of effective treatments since this disease abolishes the quality of patients' lives. In the current study, the effects of Bome Morphogenetic Protein-9 (BMP-9), Raloxifene (Ral) and Pluronic F-68 (PLF-68) were tested to investigate their potentials in treatment of OA. The *in vitro* OA model showing the characteristic biological changes (i.e. increase in wet weight, decrease in the collagen and sGAG content) associated with the OA was established with Aza-C treatment of rat chondrocytes embedded in agarose.

The results of BMP-9 treatments showed that this growth factor did not create any change in the cell number compared to healthy and control groups. However, 5 and 50 ng/ml doses decreased both sGAG and collagen synthesis of the osteoarthritic cells while 10 ng/ml dose increased the synthesis of collagen. In BMP groups the water absorption property which is considered as an indication of destruction of matrix elements was also obtained similar to that of control group. As estimated from all these negative changes in terms of treatment parameters, BMP-9 groups showed the lowest peak stress and aggregate modulus values among all groups. Hence, we may suggest that BMP-9 might be considered as either inefficient in treatment of OA or needing further investigation for an end result.

The potential therapeutic effect of Ral, an osteoporosis drug, was shown for the first time in the literature on OA. The best dose of treatment was 5 μM when

the matrix synthesized by the osteoarthritic cells is considered. The mechanical properties of the agarose-chondrocyte discs after treatments with Ral were also found consistent with the positive effects of Ral on matrix properties as the equilibrium aggregate modulus values were statistically indifferent than the healthy group and the peak stress values were numerically better than the control and BMP groups. Therefore, Ral holds promise for the treatment of OA.

The potential of PLF-68 for saving traumatized chondrocytes was previously shown. In this study, in addition to increased cell number of PLF-68 treated osteoarthritic chondrocytes, a statistically significant increase in the amount of collagen secreted by the cells was also documented for the first time. Treatment with PLF-68 was also resulted in statistically significant increase in peak stress and numerical increase in equilibrium aggregate modulus values compared to OA cells.

Further studies with human osteoarthritic chondrocytes and animal model studies should be carried out to conclude the potential therapeutic effects of Ral and PLF-68 on OA and to understand their mechanisms of action comprehensively. Besides, based on positive effects of Ral and PLF-68 on the osteoarthritic cells, observed in this study, combinations of these agents can also be investigated for OA treatment.

REFERENCES

Ackermann B, Steinmeyer J. Collagen biosynthesis of mechanically loaded articular cartilage explants. Osteoarthritis and Cartilage 2005; 13: 906-14.

Akagi M, Kanata S, Mori S, Itabe H. Possible involvement of the oxidized low density lipoprotein like/lectin like oxidized low density lipoprotein receptor-1 system in pathogenesis and progression of human osteoarthritis. Osteoarthritis and Cartilage 2007; 15(3): 281-90.

Akesson K. New approaches to pharmacological treatment of osteoporosis Bulletin of the World Health Organization 2003; 81: 657-64.

Allen M.R., Iwata K, Sato M, Burr D.B. Raloxifene enhances vertebral mechanical properties independent of bone density. Bone 2006; 39: 1130-35.

American College of Rheumatology Subcommittee, Recommendations for the medical management of osteoarthritis of the hip and knee Arthritis & Rheumatism 2000; 43:1905–1915.

Appleyard R.C., Burkhardt D., Ghosh P., Read R., Cake M., Swain M.V. Topographical analysis of the structural, biochemical and dynamic biomechanical properties of cartilage in an ovine model of osteoarthritis. Osteoarthritis Cartilage 2003; 11(1): 65-77.

Arner E.C. Aggrecanase-mediated cartilage degradation. Curr Opin Pharmacol 2002; 2: 322-29.

Badurski J.E., Daniluk S., Dobrenko A., Nowak N.A., Jeziernicka E.Z., Holiczer W. A significant improvement of womac and lequesne indexes after treatment with raloxifene in women with osteoarthritis. Osteoarthritis and Cartilage 2005 Suppl 1:158.

Bank R.A., Krikken M., Beekman B., Stoop R., Maroudas A., Lafeber F.P., te Koppele J.M. A simplified measurement of degradaed collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. Matrix Biol 1997; 16(5): 233-43.

Barrett-Conor E. Raloxifene: risks and benefits. Ann NY Acad Sci 2001; 949: 295-303.

Bellosta S., Baetta R., Canavesi R., Comparato C., Granata A., Monetti M., Cairoli F., Eberini .I, Puglisi L., Corsini A. Raloxifene inhibits matrix metaloproteinases expression and activity in macrophages and smooth muscle cells. Pharmacol Res 2007 (in press).

Blanco F.J., Ochs H., Schwaz H., Lotz M., Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995; 146: 75-85.

Blunk T., Appel S.B., Croft C., Courter D.L., Chieh J.J., Goepferich A., Khurana J.S., Gooch K.J. Bone morphogenetic protein 9: A potent modulator of cartilage development in vitro. Growth Factors 2003; 23(2): 71-7.

Bluteau G., Conrozier T., Mathieu P., Vignon E., Herbage D., Mallein-Gerin F. Matrix metalloproteases-1, -3, -13 and aggrecanase-1 and -2 are differentially expressed in human articular chondrocytes in experimental osteoarthritis. Biochim Biophys Acta 2001; 1526(2): 147-58.

Bobacz K., Erlacher L., Smolen J., Graninger W.B. Chondrocyte number and proteoglycan synthesis in the aging and osteoarthritic human articular cartilage. Ann Rheum Dis 2004; 63: 1618-22.

Bonassar L.J., Frank E.H., Murray J.C., Pagui C.G., Moore V.L., Lark M.W. Changes in cartilage composition and physical properties due to streptomyelisin degradation. Arthritis Rheum 1995; 38: 173-83.

Brandt K.D. Animal models of osteoarthritis. Biorheology 2002; 39(1-2), 221–35.

Brandt, K.D., Doherty, M., Lohmander, L.S. (Eds.). Osteoartrhitis, second ed. Oxford Universitiy Press, Oxford, 2003; 411–16.

Brandt K.D., Smith G.N. Jr., Simon L.S. Intraarticular injection of hyaluronan as treatment for knee osteoarthritis: what is the evidence? Arthritis Rheum. 2000; 43(6): 1192-203.

Brocklehurst R., Bayliss M.T., Maroudas A., Caoysh H.I., Freeman M.A.R., Revel P.A. The composition of normal and osteoarthritic articular cartilage from human knee joints. With specificial reference to unicompartmental replacement and osteoomy of the knee. J Bone Joint Surg Am 1984; 66: 95-106.

Buschmann M.D., Gluzband Y.A., Grodzinsky A.J., Hunziker E.B. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J. Cell Sci. 1995; 108: 1497-1506.

Chen T.H., Chen L., Hsieh M.S., Chang C.P., Chou D.T., Tsai S.H. Evidence for a protective role for adinopectin in osteoarthritis. Biochim Biophys Acta 2006; 1762: 711-18.

Chen Y.L., Lee H.P., Chan H.Y., Sung L.Y., Chen H.C., Hu Y.C. Composite chondroitin-6-sulfate/dermatan sulfate/chitosan scaffolds for cartilage tissue engineering. Biomaterials 2007; 2294-2305.

Christgau S., Tanko L.B., Cloos P.A.C., Mouritzen U., Christiansen C., Delaisse J.M., Hoegh-Andersen P. Suppression of elevated cartilage turnover in postmenopausal women and in ovariectomized rats by estrogen and a selective estrogen-receptor modulator (SERM) Menopause J North Am Menopause Soc 2004; 11(5): 508-18

Chubinskaya S., Kuettner K.E. Regulation of osteogenic proteins by chondrocytes. Int J Biochem Cell Biol 2003; 35: 1323-40.

Cortial D., Gouttenoire J., Rousseau C.F., Ronziere M.C., Piccardi N., Msika P., Herbage D., Mallein-Gerin F. Activation of IL-1 of bovine chondrocytes in culture within a 3D collagen-based scaffold. An in vitro model to address the effect of compounds with therapeutic potential in osteoarthritis. Osteoarthitis and Cartilage 2006; 14: 631-640.

Daniluk S., Badurski J.E., Dobrenko A., Jeziernicka E.Z., Nowak N.A., Buslowska J., Holiczer W. The inhibition of cartilage degradation markers excretion after treatment with raloxifene in women with knee osteoarthritis. Osteoarthritis and Cartilage 2005 Suppl 1:S43.

De Croos J.N.A., Dhaliwal S.S., Grynpas M.D., Pilliar R.M., Kandel R.A. Cyclic compressive mechanical stimulation induces sequential catabolic and anabolic gene changes in chondrocytes resulting in increased extracellular matrix accumulation. Matrix Biol 2006; 25: 323-31.

De Freitas P.S., Wirz D., Stolz M., Göpfert B., Friederich N.F., Daniels A.U. Pulsatile dynamic stiffness of cartilage-like materials and use of agarose gels to validate mechanical methods and models. Biomed Mater Res Part B 2006; 78B: 347-57.

D'Elia H.F., Christgau S., Mattsson L., Saxne T., Ohlsson C., Nordborg E., Carlsten H. Hormone replacement therapy, calcium and vitamin D_3 versus calcium and vitamin D_3 alone decreases markers of cartilage and bone metabolism in rheumatoid arthritis: a randomized controlled Trial Arthritis Res 2004; 6: R457-68

Démarteau O., Wendt D., Braccini A., Jakob M., Schäfer D., Heberer M., Martin I. Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes. Biochem Biophys Res Commun 2003; 310(2): 580-88.

Dodge G.R., Jimenez S.A. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis and Cartilage 2003; 11:424-32.

Dozin B., Malpeli M., Camardella L., Cancedda R., Pietrangelo A. Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines; molecular and cellular aspects. Matrix Biol 2002; 21:449-59.

El Hajjaji H., Williams J.M., Devogelaer J.P., Lenz M.E., Thonar E.J., Manicourt D.H. Treatment with calcitonin prevents the net loss of collagen, hyaluronan and proteoglycan aggregates from cartilage in the early stages of canine experimental osteoarthritis. Osteoarthritis Cartilage. 2004; 12(11):904-11.

Erlacher L., Ng C., Ullrich R., Krieger S., Luyten F. Presence of cartilage derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro. Arthrit Rheum 1998; 4(2): 263-73.

Eyrich D., Brandl F., Appel B., Wiese H., Maier G., Wenzel M., Staudenmaier R., Goepferich A., Blunk T. Long-term stable fibrin gels for cartilage engineering. Biomaterials 2007; 1: 55-65.

Farndale R.W., Buttle D.J., Barrett A.J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta. 1986; 883(2): 173-7.

Felson D.T., Zhang Y. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. Arthritis & Rheumatism 1998; 41: 1343-1355.

Flechtenmacher J., Huch K., Thonar E.J., Mollenhauer J.A., Davies S.R., Schmid T.M., Puhl W., Sampath T.K., Aydelotte M.B., Kuettner K.E. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum. 1996; 39(11):1896-904.

Forsyth C.B., Pulai J., Loeser R.F. Fibronectin fragments and blocking antibodies to α2β1 and α5β1 integrins increase collagenase 3 (matrix metalloproteinase 13) productions by human articular chondrocytes. Arthritis Rheum 2002; 46: 2368-76.

Gannon F.H., Sokoloff L. Histomorphometry of the aging human patella: histologic criteria and controls. Osteoarthr Cartil 1999; 7: 173-81.

Gay S., Kuchen S., Gay R.E., Neidhart M. Cartilage destruction in rheumatoid arthritis. Ann Rheum Dis 2002; 61 (Suppl 2): ii87.

Gebhard P.M., Gehrsitz A., Bau B., Soder S., Eger W., Aigner T. Quantification of expression levels of cellular differentiation markers does not support a shift in the cellular phenotype of osteoarthritic chondrocytes. J Orthop Res 2003; 21: 96-101.

Glass G.G. Osteoarthritis. Dis Mon 2006; 52: 343-62.

Gordon Neil F. Arthritis Your Complete Exercise Guide, Human Kinetics Publishers, USA, 1993.

Hall A.C., Horwitz E.R., Wilkins R.J. The cellular physiology of articular cartialge. Exp Physiol 1996; 81: 535-45.

Hashimoto S., Takahashi K., Amiel D., Coutts R.D., Lotz M. Chondrocyte apoptosis and nitric acid production during experimentally induced osteoarthritis. Arthritis Rheum 1998; 41: 1266-74.

Hellung-Larsen P., Assaad F., Pankratova S., Saietz B.L., Skovgaard L.T. Effects of pluronic F-68 on tetrahymena cells: protection against chemical and physical stress and prolongation of survival under toxic conditions. J Biotechnol 2000; 76: 185-95.

Hidaka C., Goodrich L.R., Chen C.T., Warren R.F., Crystal R.G., Nixon A.J. Acceleration of cartilage repair by genetically modified chondrocytes over expressing bone morphogenetic protein-7. J Orthop Res 2003; 21(4): 573-83.

Hills B.A., Crawford R.W., Phil D. Normal and prosthetic synovial joints are lubricated by surface-active phospholipids. J Arthroplasty 2003; 18(4); 499-505.

Hills R.L., Belanger L.M., Morris E.A. Bone morphogenetic protein 9 is a potent anabolic factor for juvenile bovine cartilage, but not adult cartilage. J Ortohop Res 2005; 23: 611-17.

Ho M.L., Chang J.K., Wu S.C., Chung Y.H., Chen C.H., Hung S.H., Wang G.J. A novel terminal differentiation model of human articular chondrocytes in three dimensional cultures mimicking chondrocyte changes in osteoarthritis. Cell Biol Int 2006; 30(3):288-294.

Høegh-Andersen P., Tankó L.B., Andersen T.L., Lundberg C.V., Mo J.A., Heegaard A.M., Delaissé J.M., Christgau S. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application Arthritis Res Ther 2004; 6: R169-R180.

Hoemann C.D., Sun J., Légaré A., McKee M.D., Buschmann M.D. Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle. Osteoarthritis and Cartilage 2005; 13(4): 318-329.

Homandberg G.A., Guo D., Ray C.L.S., Ding L.B.S. Mixtures of glucosamine and chondroitin sulfate reverse fibronectin fragment mediated damage to cartilage more effectively than either agent alone. 2006; Osteoarthritis and Cartilage 14: 793-806.

Homandberg G.A., Hui F., Wen C. Fibronectin fragment mediated cartilage chondrolysis: Suppression by antioxidants. Biochim Biophys Acta 1996; 1317: 134-42.

Homandberg G.A., Kang Y., Zhang J., Cole A.A., Williams J. M. A single injection of fibronectin into the rabbit knee joints enhances catabolism in the articular cartilage followed by reparative responses but also induces systematic effects in the noninjected joints. Osteoarthritis Cartilage 2001; 9: 673-83.

Homandberg G.A., Meyers R., Williams J. Intraarticular injection of fibronectin fragments causes severe depletion of cartilage proteoglycans in vivo. J Rheumatol 1993; 20: 1378-82.

Huser C.A., Peacock M., Davies M.E. Inhibition of caspase-9 reduces chondrocyte apoptosis and proteoglycan loss following mechanical trauma. Osteoarthritis and Cartilage 2006; 14: 1002-10.

Hyttinen M.M., Toyras J., Lapvetelainen T., Lindblom J., Prockop D.J., Li S.W., Arita M., Jurvelin J.S., Helminen H.J. Inactivation of one allele of the type II collagen gene alters the collagen network in murine articular cartilage and makes cartilage softer. Ann Rheum Dis 2001; 60: 262–68.

Ikenou T., Trindade M.C.D., Lee M.S., Lin E.Y., Schurman D.J., Goodman S.B., Smith R.L. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. J Orthop Res 2003; 21: 110-16.

Karlson E.W., Mandl L.A., Aweh G.N., Sangha O., Liang M.H., Grodstein F. Total hip replacement due to osteoarthritis: the importance of age, obesity, and other modifiable risk factors. Am J Med. 2003; 114(2): 158-9.

Kim H.A., Lee Y.J., Seong S.C., Choe K.W., Song Y.W. Apoptotic chondrocyte death in human osteoarthritis . J Rheumatol 2000; 27: 455-62.

Korhonen R.K., Laasanen M.S., Töyras J., Lappalainen R., Helminen H.J., Jurvelin J.S. Fibril reinforced poroelastic model predicts spesifically mechanical behavior of normal, proteoglycan depleted and collagen degraded artcular cartilage. J Biomech. 2003; 9: 1373-79.

Kuettner, K.E., Goldberg, V.M., Ed., Osteoarthritic Disorders, The Academy, Rosemont, IL, 1995.

Kuettner, K.E., Schleyerbach, R., Peyron, J.G., Hascall, V.C. Articular Cartilage and Osteoarthritis, Raven Press, New York, 1991.

Lafeber F.P., van der Kraan P.M., Huber-Bruning O., van den Berg W.B., Bijlsma J.W. Osteoarthritic human cartilage is more sensitive to transforming growth factor beta than is normal cartilage. Br J Rheum 1993; 32: 281-6.

Lee C.S.D., Gleghorn J.P., Choi N.W., Cabodi M., Stroock A.D., Bonassar L.J. Integration of layered chondrocyte-seeded alginate hydrogel scaffolds. Biomaterials 2007; 28 2987–93.

Lee R.C., Hannig J., Matthews K.L., Myerov A., Chen C.T. Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. Ann N Y Acad Sci. 1999; 888: 266-73.

Leeb B.F., Schweitzer H., Montag K., Smolen J.S. A metaanalysis of chondroitin sulfate in the treatment of osteoarthritis. J Rheumatol 2000; 27: 205-11.

Leroux M.A., Cheung H.S., Bau J.L., Wang J.Y., Howel D.S., Setton L.A. Altered mechanics and histomorphometry of canine tibial cartilage following joint immobilization. Osteoarthritis and Cartilage 2001; 9: 633– 40.

Li X., Jin L., Balian G., Laurencin C.T., Anderson D.G. Demineralized bone matrix gelatin as scaffold for osteochondral tissue engineering. Biomaterials 2006; 27(11): 2426-2433

Lorenz H., Richter W. Osteoarthritis: Cellular and molecular changes in degeneratingcartilage. Prog Histochem Cytochem 2006; 40: 135-63.

Lorenz H., Wenz W., Ivancic M., Steck E., Richter W. Early and stable upregulation of collagen type II, collagen type I and YKL40 expression levels in cartilage during early experimental osteoarthritis occurs independent of joint location and histological grading. Arthritis Res Ther 2005; 7(1): R156-65.

Lufkin E.G., Whitaker M.D., Nickelsen T., Argueta R., Caplan R.H., Knickerbocker R.K. Treatment of established postmenopausal osteporosis with raloxifene: a randomized trial. J Bone Miner Res 1998; 13: 1747-54.

Luyten F., Chen P., Paralker V., Reddi H. Recombinant bone morphogenetic protein-4, transforming growth factor-beta 1, and activin A enhance the cartilage phenotype of articular chondrocytes in vitro. Exp Cell Res 1994; 210(2): 224-29.

Luyten, F.P., Cunningham N.S., Ma S., Muthukumaran N., Hammonds R.G., Nevins W.B., Woods W.L., Reddi A H. Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J Biol Chem 1989; 264(23): 13377-80.

Lübke C., Ringe J., Krenn V., Fernah G., Pelz S., Kreusch-Brinker R., Sittinger M., Psulitschke M. Growth characterization of neo porcine cartilage pellets and their use in an interactive culture model. Osteoarthritis and Cartilage 2005; 13: 478-87.

Majumdar M.K., Wang E., Morris E.A. BMP-2 and BMP-9 promote chondrogenic differentiation of human multipotential mesenchymal cells and overcome the inhibitory effect of IL-1. J Cellular Physiol 2001; 189: 275-84.

Mankin H.J., Dorfman Scheck M., Sakovich L. Degenerative joint disease of the canine hip: experimental production by multiple papain and prednisone injections. Clin. Orthop. Relat. Res. 1972; 86: 115–20.

Mankin H.J., Thrasher A.Z. Water content and binding in normal and osteoarthritic human cartilage. J Bone Joint Surg 1975; 57-A:76-80.

Marks J.D., Pan C.Y., Bushell T., Cromie W., Lee R.C. Amphiphilic, tri-block copolymers provide potent membrane-targeted neuroprotection. FASEB J. 2001; 15(6): 1107-9.

Martel-Pelletier J. Pathophysiology of osteoarthritis. Osteoarthr. Cartil. 12 (Suppl. A), 2004; S31–33.

Maskarinec S.A., Hannig J., Lee R.C., Lee K.Y. Direct observation of poloxamer 188 insertion into lipid monolayers. Biophys J. 2002; 82(3):1453-9

Mauck R.L., Nicoll S.B., Seyhan S.L., Ateshian G.A., Hunk C.T. Synergistic action of growth factors and dynamic loading for articular cartilage tissue enginering. Tissue Engineering 2003; 9(4): 597-611.

Mauck R.L., Soltz M.A., Wang C.C.B., Wong D.D., Chao P.H.G.C., Valhmu W.B., Hung C.T., Ateshian G.A. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. Trans ASME 2000; 122: 252-60.

Mauck R.L, Wang C.C.B., Oswald E.S., Ateshian G.A., Hung C.T. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis and Cartilage 2003; 11: 879-90.

McAlindon T.E., LaValley M.P., Gulin J.P., Felson D.T. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and metanalysis. JAMA 2000; 283: 1469-75.

Mizuno S., Tateishi T., Ushida T., Glowacki J. Hydrostatic pressure enhances matrix synthesis and accumulation by bovine chondrocytes in three-dimensional culture. J Cellular Physiol 2002; 193: 319-27.

Mizuta H., Kudo S., Nakamura E., Otsuka Y., Takagi K., Hiraki Y. Active proliferation of mesenchymal stem cells prior to chondrogenic repair response in full thickness defects of articular cartilage. Osteoarthritis and Cartilage 2004; 12: 586-96.

Moody H.R., Brown C P , Bowden J.C., Crawford R.W., McElwain D.L.S., Oleyede A. O. In vitro degradation of articular cartilage: does trypsin teatment produce consistent results. J Anat. 2006; 209: 259-67.

Mouw J.K., Case N.D., Guldberg R.E., Plaas A.H.K., Levenston M.E. Variations in matrix composition and GAG fine structure among scaffolds for cartilage tissue engineering. Osteoarthritis and Cartilage 2005; 13: 828-36.

Murphy J.M., Fink D.J., Hunzinker E.B., Barry F.P. Stem cell therapy in a caprine model of osteoarthritis. Arthrit & Rheum 2003; 48(12). 3464-74.

Murrel G.A., Jang D., Williams R.J. Nitric oxide activates metalloprotease enzymes in articular cartilage. Biochim Biophys Res Commun 1995; 206: 15-21.

Na K., Park J.H., Sun B.K., Woo D.G., Chung H.M., Park K.H. Delivery of dexamethasone, ascorbate, and growth factor (TGFβ3) in thermoreversible hydrogel constructs embedded with rabbit chondrocytes. Bioamterials 2006; 5951-57.

National Institute of Health Sciences, 2006, http://www.nihs.go.jp/dbcb/Bio-Topic/protein.pdf. Last access date: September 2007.

Nesic D., Whiteside R., Brittberg M., Wendt D., Martin I., Mainil-Varlet P. Cartilage tissue engineering for degenerative joint disease. Adv Drug Del 2006; 58: 300-22.

Nieminen H.J., Töyras J., Rieppo J., Nieminen M.T., Hirvonen J.,Korhonen R., Jurvelin J.S. Real-time ultrasound analysis of articular cartilage degradation in vitro. Ultrasound Med Biol. 2002: 4: 519-25.

Nilsson O., Falk J., Ritzen E.M., Baron J., Savendahl L. Raloxifene acts as an estrogen agonist on the growth plate. Endocrinology 2003; 144(4):1481-85.

Nishida Y., Knudson C.B., Knudson W. Osteogenic Protein-1 inhibits matrix depletion in a hyaluronan hexasaccharide-induced model of osteoarthritis. Osteoarthritis and Cartilage 2004; 12: 374-82.

Nishida Y., Knudson C.B., Kuettner K.E., Knudson W. Osteogenic protein-1 promotes the synthesis and retention of extracelullar matrix within bovine articular cartilage and chondrocyte cultures. Osteoarthritis and Cartilage 2000; 127-36.

O'Driscoll S.W. Articular cartilage regeneration using periosteum. Clin Orthop Relat Res 1999; 186-203.

Oliveira J.M., Rodrigues M.T., Silva S.S., Malafaya P.B., Gomes M.E., Viegas C.A., Dias I.R., Azevedo J.T., Mano J.F., Reis R.L. Novel hydroxyapatite/chitosan bilayered sacoffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells. Biomaterials 2006; 27: 6123-6137.

Oliveria S.A., Felson D.T., Cirillo P.A., Reed J.L., Walker A.M. Body weight, body mass index, and incident symptomatic osteoarthritis of the hand, hip, and knee. Epidemiology 1999; 10(2): 161-66.

Ospelt C., Neidhart M., Gay R.E., Gay S. Synovial activation in rheumatoid arthritis. Front Biosci 2004, 9: 2323-34.

Ozgocmen S., Kaya H., Fadillioglu E., Yılmaz Z. Effects of calcitonin, risedronate and raloxifene on erythrocyte antioxidant enzyme activity, lipid peroxidation, and nitric oxide in postmenapousal osteoporosis. Arch Med Res 2007; 38: 196-205.

Patwari P., Cook M.N., Micco M.A., Blake S.M., James I.E., Kumar S., Cole A.A., Lark M.W. Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum 2003; 48: 1292-1301.

Phillips D.M., Haut R.C. The use of a non-ionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants J Orthop Res 2004; 22(5):1135-1142.

Piscoya J.L., Fermor B., Kraus V.B., Stabler T.V., Gullak F. The influence of mechanical compression on the induction of osteoarthritis related biomarkers in articular cartilage explants. Osteoarthritis and Cartilage 2005; 13: 1092-99.

Prestwood K.M., Gunness M., Mauchmore D.B., Lu Y., Wong M., Raisz L.G. A comparison of the effects of raloxifene and estrogen on bone in postmenapousal women. J Clin Endocrinol Metab 2000; 85: 2197-202.
Quinn T.M., Grodzinsky A.J., Byschmann M.D., Kim Y.J., Hunzinker E.B. Mechanical compression alters proteoglycan deposition and matrix deformation around individual cells in cartilage explants. J Cell SCİ 1998; 111; 573-583.

Reid I.R., Eastell R., Fogelman I., Adachi J.D., Rosen A., Netelenbos C., Watts N.B., Seeman E., Ciaccia A.V., Draper M.W. A comparison of the effects of raloxifene and conjugated equine estrogen on bone and lipids in healthy postmenapousal women. Arch Intern Med 2004; 164: 871-79.

Richette P., Dumontier M.F., Tahiri K., Widerak M., Torre A., Benallaoua M., Rannou F., Corvol M.T., Savouret J.F. Oestregens inhibit interleukin 1β-mediated nitric oxide synthase expression in articular chondrocytes through nuclear factor-kappa B impairement. Ann Rheum Dis 2007; 66(3): 345-50.

Richy F., Bruyere O., Elthgen O., Cucherat M., Henrotin Y., Reginster J.Y. Structural and symptomatic efficacy of glucosamine and chondroitin in knee osteoarthritis. 2003; 163: 1514-22.

Rieppo J., Töyräs J., Nieminen M.T., Kovanen V., Hyttinen M.M., Korhonen R.K., Jurvelin J.S., Helminen H.J. Structure-function relationships in enzymatically modified articular cartilage. Cells Tissues Organs 2003; 175(3): 121-32.

Robert L.M., Steven B.N., Sara S., Gerard A.A., Clark T.H. Synergistic Action of Growth Factors and Dynamic Loading for Articular Cartilage Tissue Engineering. Tissue Eng 2003; 9(4): 597-611.

Rogers A., Clowes C.A., Eastell P.R. Different effects of raloxifene and estrogen on interleukin β1 and interleukin-1 receptor antagonist production using in vitro and ex vivo studies. Bone 2007; 40: 105-10.

Roos H., Adalberth T., Dahlberg L., Lohmander L.S. Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthr Cartil 1995; 3: 261–67.

Rundell S.A., Baars D.C., Phillips D.M., Haut R.C. The limitation of acute necrosis in retro-patellar cartilage after a severe blunt impact to the in vivo rabbit patello-femoral joint J Orthop Res 2005; 23(6):1363-1369.

Sailor L.Z., Hewick R.M., Morris E.A. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. J Orthop Res 1996; 14(6): 937-45.

Salva C., Piganet A., Humbert L., Berenbaum F., Thiron S. Immature murine articular chondrocytes in primary culture: a new tool for investigating cartilage. Osteoarthitis and Cartilage 2005; 13: 243-249.

Scheck M., Sakovich L. Degenerative joint disease of the canine hip: experimental production by multiple papain and prednisone injections. Clin Orthop Relat Res. 1972; 86: 115-20.

Seemayer C.A., Neidhart M., Jüngel A., Gay R.E., Gay S. Synovial fibroblasts in joint destruction of rheumatoid arthritis. Drug Discovery Today: Disease Mechanisms 2005; 2(3): 359-65.

Shagemann J.C., Mrosek E.H., Landers E.H., Kurz H., Erggelet C. Morphology and function of ovine articular cartilage chondrocytes in 3-D hydrogel culture. Cells Tissues Organs 2006; 182: 89-97.

Sherwood J.L., Riley S.L., Palazzolo R., Brown S.C., Monkhouse D.C., Cotes M., Griffith L.G., Landeen L.K., Ratcliffe A. A three-dimensional osteochondral composite scaffold for articular cartilage repair. Biomaterials 2002; 23: 4739-51.

Shui C., Scutt A. Mild heat shock induces proliferation, alkaline phosphatase activity, and mineralization in human bone marrow stromal cells and Mg-63 cells in vitro. J Bone Mineral Res 2001; 16(4): 731-41.

Solchaga L.A., Temenoff J.S., Gao J., Mikos A.G., Caplan A.I., Goldberg V.M. Repair of osteochondral defects with hyaluronan- and polyester-based scaffolds. Osteoarthritis and Cartilage 2006; 13: 297-309.

Stanford C.M., Jacobson P.A., Eanes E.D., Lemabke L.A., Midura R.J. Rapidly forming apatitic mineral in an osteoblast cell line (UMR 106-01 BSP). J Biol Chem 270: 9420-8.

Stanton H., Ung L., Fosang A.J. The 45 kDa collagen-binding fragment of fibronectin induces matrix metalloproteinase-13 synthesis by chondrocytes and aggrecan degradation by aggrecanases. Biochem J. 2002; 364(Pt 1): 181-90.

Stöve J., Schneider-Wald B., Scharf H.P., Schwarz M.L. Bone morphogenetic protein 7 (bmp-7) stimulates proteoglycan synthseis in human osteoarthritic chondrocytes in vitro. Biomed Pharmacother 2006; 60: 639-43.

Studer R., Jaffurs D., Stefanovic-Racic M., Robbins P.D., Evans C.H. Nitric oxide in osteoarthritis. Osteoarthritis and Cartilage 1999; 7: 377-79.

Suh J.K., Aoren A., Muzzonigro T.S., Disilvestro M., Fu F.H. Injury and repair and articular cartilage: scientific issues. Operative Techniques in Orthopaedics 1997; 7: 270-78.

Swoboda B. Epidemiological arthrosis research. Orthopade 2001; 11: 834-40.

Taguchi T., Sawabe Y., Kobayashi H., Moriyoshi Y., Kataoka K., Tanaka J. Preparation and characterization of osteochondral scaffold. Materials Science and Engineering: C 2004; 24(6-8): 881-885

Tallheden T., Bengstsson C., Bransting C., Sjogren-Jansson E., Carlsson L., Peterson M., Brittberg M., Lindahl A. Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. Arthritis Res Ther 2005: 7: 560-68.

Tallheden T., Karlsson C., Brunner A., van der Lee J., Hagg R., Tommashi R., Lindahl A. Gene expression during redifferentiation of human articular chondrocytes. Osteoarthritis and Cartilage 2004; 12: 525-35.

Tamai N., Myoui A., Hirao M., Kaito T., Ochi T., Tanaka J., Takaoka K., Yoshikawa H. A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis and Cartilage 2005; 13: 405-17.

Tchetina R., Ono M., Squires G., Poole A.R. Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular lesions. J Rheumotol 2005; 32(5): 876-86.

Toyoda T., Seedhom B.B., Yao J.Q., Kirkham J., Brookes S., Bonass W.A. Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose. Arth Rheumat 2003; 48(10): 2865-72.

Trindade M.C.D., Shida J.I., Takashi I., Lee M.S., Lin E.Y., Yaszay B., Yerby S., Smith R.L. Intermittent hydrostatic pressure inhibits matrix metalloproteinase and pro-inflammatory mediator release from human osteoarthritic chondrocytes in vitro. Osteoarthritis and Cartilage 2004; 12: 729-35.

Urist M.R. Bone formation by autoinduction Science 1965; 50: 893-99.

Vanwanseele B., Lucchinetti E., Stussi E. The effects of immobilization on the characteristics of articular cartilage: current concepts and future directions. Osteoarthritis and Cartilage 2002; 10: 408–19.

Vogel V.G., Costantino J.P., Wickerham D.L., Cronin W.M., Cecchini R.S., Atkins J.N. Bevers T.B., Fehrenbacher L., Pajon E.R. Jr., Wade J.L. 3rd, Robidoux A., Margolese R.G., James J., Lippman S.M., Runowicz C.D., Ganz P.A., Reis S.E., McCaskill-Stevens W., Ford L.G., Jordan V.C., Wolmark N. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes. JAMA 2006; 295(23): 2727-41.

Wakitani S., Imoto K., Yamamoto T., Saito M., Murata N., Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis and Cartilage 2002; 10: 199-206.

Weisser J., Rahfoth B., Timmermann A., Aigner T., Brauer R., Vonder Mark K. Role of growth factors in rabbit aricular cartilage repair by chondrocytes in agarose. Osteoarthritis and Cartilage 2001; 9 (Suppl): S48-54.

Wong M., Siegrist M., Goodwin K. Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. Bone 2003; 33: 685-93.

Wozney J.M., Rosen V., Celeste A.J., Mitsock L.M., Whitters M.J., Kriz R.W., Hewick R.M., Wang E.A. Novel regulators of bone formation: molecular clones and activities. Science 1988; 242(4885): 1528-34.

Wozney J.M. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 1992; 32(2): 160-67.

Yang I.H., Kim Y.H., Sun H.J., Kim S.J., Lee J.W. Comparison of phenotypic characterization between alginate and pellet culture systems as chondrogenic differentiation models for human mesenchymal stem cells. Yonsei Med J 2004; 45(5): 891-900.

Yeh T.T., Wu S.S., Lee C.H., Wen Z.H., Lee H.S., Yang Z., Nimni M.E., Han B. The short term therapeutic effect of recombinant human bone morphogenetic protein-2 on collagenase-induced lumbar facet joint osteoarthritis in rats. Osteoarthritis and Cartilage 2007 (in press).

APPENDIX A

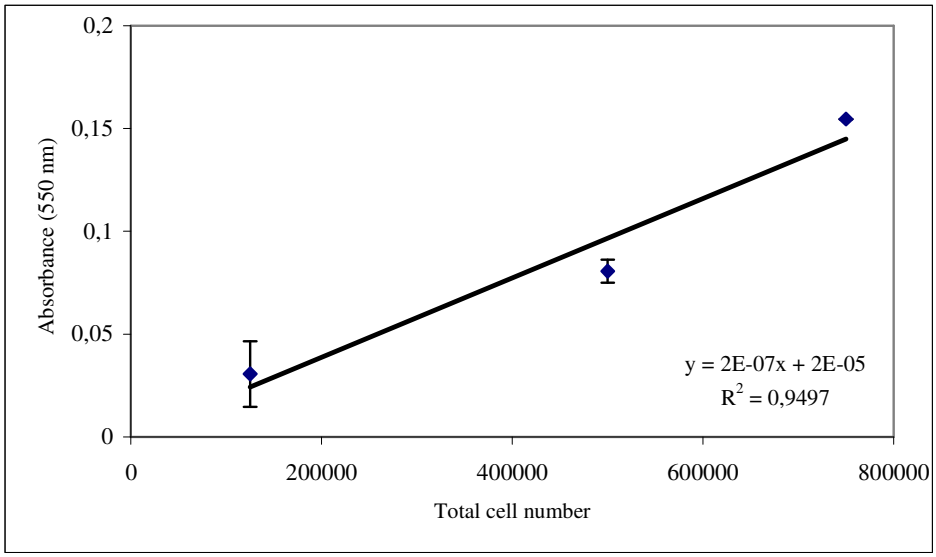


Figure A.1. Calibration curve of agarose-chondrocyte discs using MTT assay.

APPENDIX B

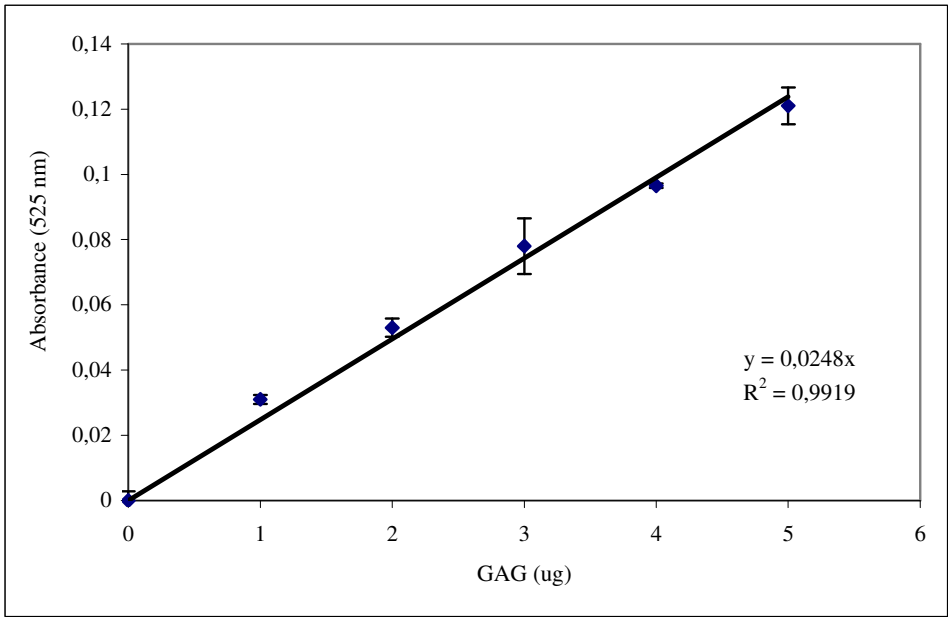


Figure B.1 Calibration curve for GAG determination using DMMB assay.

APPENDIX C

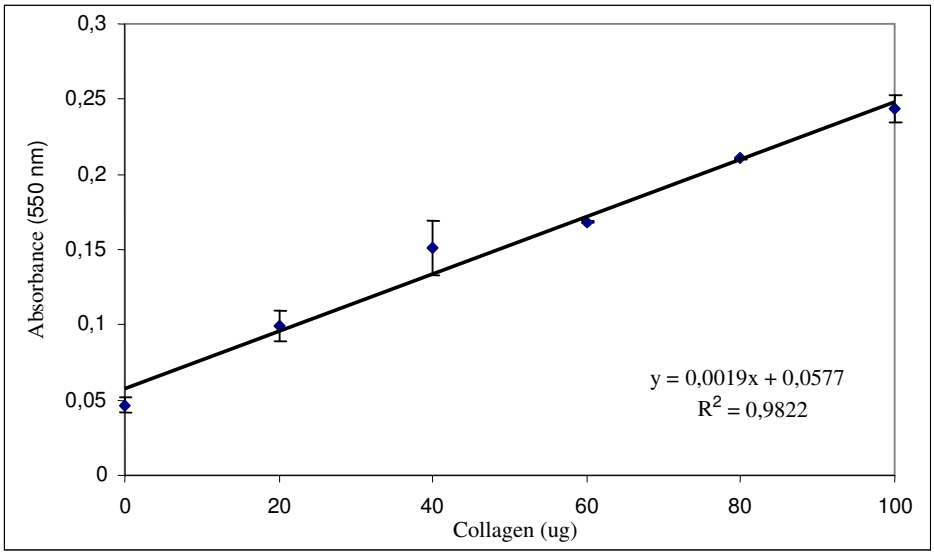


Figure C.1 Calibration curve for collagen determination using Sirius Red S assay.

APPENDIX D

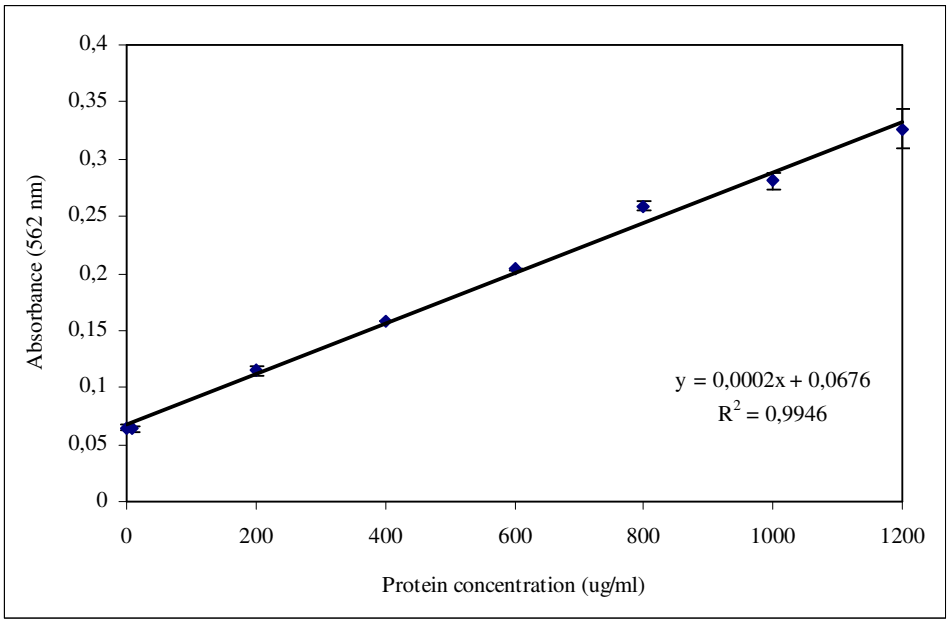


Figure D.1. Calibration curve for protein quantitation using BCA assay.