

CARTILAGE REGENERATION VIA SMALL MOLECULE INDUCED RAT BONE
MARROW MESENCHYMAL STEM CELLS ON NATURAL BIOMATERIALS



by
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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University

2021

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor Prof. Dr. Gamze Köse for her encouragement, support, and constructive suggestions during this study, as well as my whole undergraduate and postgraduate years. It was a privilege to work and study under her guidance.

I am grateful to Prof. Dr. Fatma Neşe Kök for her valuable recommendations and contributions to this study. I also would like to thank her for helping me to find alternative solutions to overcome some of the difficulties I encountered throughout the study.

I would also like to extend my special thanks to Assoc. Prof. Dr. Ali Özhan Aytekin for his suggestions at the beginning of the study, and Assist. Prof. Dr. Görke Gürel Peközer for her help during the development of the study and some of the experimental work. I also thankful to Dr. Nergis Abay Akar for being a part of this study. I also would like to appreciate to Assoc. Prof. Ayşegül Kuşkucu and my thesis committee members for their kindness and support. I would like to thank TÜBİTAK-MAG (Project no:117M287) for funding this project.

I thank to Yeditepe University Tissue Engineering Group members: Assist. Prof. Ayşe Ceren Çalıkoğlu Koyuncu, Dr. İrem Ayşe Kanneçi Altınışik, Dr. Görkem Özdemirli, Seçil Demir, Begüm Okutan, İrem Büyük, Didem Aksu, Kardelen Tüfekçi and Fatma Çayır for being such kind and supportive friends. I am very grateful to my officemates for giving me the moral support at the final stages of my PhD work. I wish to express my special gratitude to my friend Dr. Ayşegül Atasoy Zeybek for her unconditional support and cherish, even if we are not in the same country anymore. I also owe my special thanks to my dearest friend Aysu Bilge Gökyüzü for giving me the insights both spiritually and scientifically.

I would like to express my deep sense of gratitude to my best friends Pınar Çınar, Dr. Halenur Şahin Mahmutoğulları, Dr. Özge Özgenç Çınar and Emine Topaloğlu Bircan for being by my

side in every aspect of my life. Besides, I am very grateful to Dr. Özge Özgenç Çınar for her assistance in the histological studies of this study.

Last but not least, I am extremely grateful for having such a wonderful family; especially my dearest parents Ahmet and Şenay, and my younger brother Gökem. I could not succeed without their encouragement, support, love and patience in every stage of my life. I am also highly indebted to my dear husband, Necati Barış Taş, for his love, understanding, help, and unconditional support in every single moment of our life together.

ABSTRACT

CARTILAGE REGENERATION VIA SMALL MOLECULE INDUCED RAT BONE MARROW MESENCHYMAL STEM CELLS ON NATURAL BIOMATERIALS

Cartilage is an avascular and aneural tissue that has low self-repair capacity upon damages resulted from diseases and injuries. Current orthopaedic treatments have some limitations such as donor site morbidity, and inadequate mechanical and functional properties of the newly formed tissue compared to native tissue. In this context, cartilage tissue engineering can offer an alternative solution to overcome the limitations of conventional orthopaedic treatments. In this study, natural biomaterials Locust bean gum (LBG), Xanthan gum (XG) and Mastic Gum (MG) were combined to form cryogel scaffolds for cartilage tissue engineering. The LBG-XG and LBG-XG-MG cryogels were physically, mechanically, and chemically characterized. The results demonstrated the macro-porous, and interconnected pore structures of the cryogels and sustained release of the small molecule Kartogenin (KGN) from cryogels to induce chondrogenic differentiation. Furthermore, these cryogels showed similar mechanical properties with the native cartilage tissue and other polysaccharide-based cryogels in the literature. According to cell viability analysis, rat bone marrow mesenchymal stem cells (RBM-MSCs) were able to attach, grow, and proliferate inside the cryogels. The cryogels having concentrations of 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG were selected with respect to the results of characterizations and cell viability analysis. Further *in vitro* stainings and quantitative real time polymerase chain reaction analysis revealed that RBM-MSCs produced cartilage specific matrix molecules and expressed cartilage specific genes inside the selected cryogels. *In vivo* studies were performed in an ectopic rat model to test the chondrogenic differentiation. The RBM-MSCs were differentiated into chondrocyte like cells in KGN-loaded cryogels after 4 and 8 weeks of implantation. All these results showed that LBG-XG and LBG-XG-MG cryogels have a potential to be used in cartilage tissue engineering.

ÖZET

DOĞAL BİYOMALZEMELER ÜZERİNDEKİ KÜÇÜK MOLEKÜLE İNDÜKLENMİŞ SIÇAN KEMİK İLİĞİ KÖK HÜCRELERİ İLE KIKIRDAK YENİLENMESİ

Kıkırdak, hastalık ve yaralanmalardan kaynaklanan hasarlarda kendini yenileme kapasitesi düşük olan, damar ve nöral doku içermeyen bir dokudur. Mevcut ortopedik tedaviler, donör bölge morbiditesi ve doğal dokuya kıyasla yeni oluşan dokunun yetersiz mekanik ve fonksiyonel özelliklere sahip olması gibi bazı limitasyonlara sahiptir. Bu bağlamda, kıkırdak doku mühendisliği, geleneksel ortopedik tedavilerin limitasyonlarının aşılabilmesi için alternatif bir çözüm sunabilir. Bu çalışmada kıkırdak doku mühendisliği için, doğal biyomateryaller olan Keçiboynuzu gamı (KBG), Ksantan gamı (KG) ve Damla Sakızı (DS) birleştirilerek kriyojel doku iskeleri oluşturulmuştur. KBG-XG ve KBG-XG-DS kriyojelleri fiziksel, mekanik ve kimyasal olarak karakterize edilmiştir. Sonuçlar, kriyojellerin makro gözenekli ve birbirine bağlı gözenek yapılarını ve kondrojenik farklılaşmayı indüklemek için kriyojellerden Kartogenin (KGN) küçük molekülünün sürekli salındığını göstermiştir. Ayrıca bu kriyojeller, literatürdeki doğal kıkırdak dokusu ve diğer polisakkarit bazlı kriyojellerle benzer mekanik özellikler göstermiştir. Hücre canlılığı analizine göre, sıçan kemik iliği mezenkimal kök hücreleri (SKİ-MKH'ler) kriyojellerin içinde tutunmuş, büyümüş ve çoğalmıştır. Karakterizasyon ve hücre canlılığı analizlerinin sonuçlarına göre yüzde 3 (k/h) - 1:1 (k/k) KBG-XG ve yüzde 3 (k/h) - 1:1 (k/k) KBG-XG + 5 mg/mL DS konsatrasyonlarına sahip kriyojeller seçilmiştir. Sonraki *in vitro* boyamalar ve kantitatif gerçek zamanlı polimeraz zincir reaksiyonu analizi, seçilen kriyojellerin içindeki SKİ-MKH'lerin kıkırdağa özgü matriks moleküllerini ürettiğini ve genlerini ifade ettiğini göstermiştir. Kondrojenik farklılaşmayı test etmek için ektopik sıçan modelinde *in vivo* çalışmalar yapılmıştır. SKİ-MKH'ler, KGN yüklü kriyojellerin içinde, implantasyonlarının 4. ve 8. haftalarında, kondrosit benzeri hücrelere farklılaşmıştır. Bu sonuçlar, KBG-XG ve KBG-XG-DS kriyojellerinin kıkırdak doku mühendisliğinde kullanılma potansiyeline sahip olduğunu göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

ΔL	Change in length
$^1\text{H-NMR}$	Proton nuclear magnetic resonance
A	Area
ACAN	Aggrecan
ACTB	Beta-Actin
ADMSC	Adipose derived mesenchymal stem cell
ANOVA	Analysis of variance
BC	Bacterial cellulose
BM-MSC	Bone marrow mesenchymal stem cell
BMP	Bone morphogenic protein
CBF β	Core binding factor beta subunit
CM	Chondrogenic medium
CMG	Chios mastic gum
COL1A1	Type I collagen
COL2A1	Type II collagen
D ₂ O	Deuterated water
DMA	Dynamic mechanical analyzer
DS	Damla sakızı
DSC	Differential scanning calorimetry
E	Young's modulus
ECM	Extracellular matrix
ESC	Embryonic stem cell
F	Force
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FLNA	Filamin A

FT-IR	Fourier-Transform Infrared Spectroscopy
G	Guluronic acid
GA	Glycolide
GAG	Glucosaminoglycan
GM	Growth medium
GRAS	Generally recognized as safe
HA	Hyaluronic acid
HMWHA	High molecular weight hyaluronic acid
IGF	Insulin-like growth factor
IPSC	Induced pluripotent stem cell
KBG	Keçi boynuzu gamı
KG	Ksantan gam
KGN	Kartogenin
LA	Lactide
LBG	Locust bean gum
LMWHA	Low molecular weight hyaluronic acid
Lo	Initial length
M	Mannuronic acid
M-G	Mannose to galactose
MG	Mastic gum
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NCAM	Neural cell adhesion molecule
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PEG	Poly (ethylene glycol)
PEG	Poly(ethylene glycol)

PES	Phenazine ethosulfate
PG	Proteoglycan
PGA	Poly (glycolic acid)
PLA	Poly (lactic acid)
PPF	Poly (propylene fumarate)
PUR	Polyurethane
qRT-PCR	Quantitative real time polymerase chain reaction
RBMC	Rat bone marrow cell
RBM-MSC	Rat bone marrow mesenchymal stem cells
RGD	Arg (Arginine)-Gly(Glycine)-Asp(Aspartic acid)
RHAMM	The receptor for hyaluronan mediated motility
RT	Room temperature
RUNX	Runt-related transcription factor
SD	Standard deviation
SEM	Scanning electron microscopy
SKİ-MKH	Sıçan kemik iliği mezankimal kök hücreleri
SOX9	SRY-type high mobility group box 9
Tg	Glass transition temperature
TGF- β	Transforming growth factor beta
TLR-2	Toll-like receptor 2
VEGF	Vascular endothelial growth factor
XG	Xanthan gum

1. INTRODUCTION

Tissue engineering is a developing field that has the potential to provide permanent solutions to tissue damage and tissue loss to millions of people each year. The fundamental components of the tissue engineering are cells, natural or artificial polymeric scaffolds and stimulating factors, which can be used alone or in combination [1]. In adult cartilage tissue, sparsely distributed and non-dividing chondrocytes, scarceness of progenitor cells and deprivation of vascular supply cause limited ability to self-repair [2]. Therefore, cartilage tissue is an ideal candidate for tissue engineering.

The 3-D scaffolds play a crucial role for cartilage tissue engineering applications, since they provide environment to support cell growth and reinforce the tissue regeneration [3]. In this manner, an ideal scaffold should be biocompatible, biodegradable and bioresorbable with an appropriate degradation rate that matches the speed of new tissue formation. They should also possess porous structure to enable cell migration and fluid flow for transportation of nutrients and extraction of the metabolic waste, present a suitable surface chemistry to cells for adhesion, differentiation and proliferation, and also have sufficient mechanical properties to bear the growth of tissue under certain pressure at the site of implantation [4,5].

The aim of this study was to design tissue engineering constructs to promote chondrogenic differentiation of rat bone marrow mesenchymal stem cells (RBM-MSCs) both *in vitro* and *in vivo*. In this context three natural materials; Locust Bean Gum, Xanthan Gum and Mastic Gum were used to prepare cryogels to support RBM-MSCs during differentiation process. Small molecule KGN was entrapped in these cryogels in order to induce chondrogenic differentiation. The chondrogenic capability of these tissue engineering constructs were investigated throughout the research.

2. THEORETICAL BACKGROUND

2.1. CARTILAGE

Cartilage is a supporting skeletal tissue composed of cells surrounded by an extracellular matrix (ECM) that may undergo mineralization process depending on the type of cartilage found in different species or organisms. It is an avascular tissue which lack of nerves unlike bone tissue. The nutrient uptake occurs by diffusion because of the absence of blood vessels. During fetal development, cartilage acts as a template for the skeleton, later through the adulthood it remains in ribs, ears, trachea, bronchi and articular surfaces. In structural manner, cartilage has ability to resist and absorb compressive and tensile forces. Functionally, it has significant roles in skeletal growth, repair and development, articulation and maintaining patency of the respiratory tract [6,7].

Cartilage is categorized as hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline cartilage is found on articulating surfaces of bones (articular cartilage), between ribs and sternum (costal cartilage), walls of trachea and larger bronchial tubes, and external part of nose [8]. Articular cartilage is the most predominant type of hyaline cartilage that gives ability to joints to bear shear forces and loads nearly 20 times of the body weight [9]. It is formed by chondrocytes, which are the only cell type present in the cartilage. The initial steps of cartilage formation are mesenchymal stem cell (MSC) migration, condensation and differentiation. Cell-cell and cell-matrix interactions direct progenitor MSC recruitment. Before condensation, progenitor cells produce a small amount of ECM having hyaluronan, type I and type IIA collagen. The ECM accumulates at the center of cell mass and separates cells from each other. The cells surrounding the cell mass also produce ECM, which eventually becomes perichondrium. The chondroprogenitor cells start to synthesize versican, tenascin, syndecan, and cell adhesion molecules such as neural cadherin (N-cadherin) and neural cell adhesion molecule (NCAM), that regulates cell-cell interactions. Before chondrogenic differentiation, fibronectin binds newly synthesized syndecan, therefore decreasing NCAM production.

Following the downregulation of type I collagen, N-cadherin and fibronectin and, upregulation of thrombospondins, matrilins and tenascins, the chondroprogenitor cells start to differentiate into chondrocytes [6, 10]. Later, chondrocytes express Sry-type high-mobility group box 9 (SOX9) transcription factor and then synthesize cartilage specific ECM rich in collagen fibers (type II collagen, IX and XI) especially type II B collagen and proteoglycans (PG) such as aggrecan. Chondrocytes found in the middle of the condensation zone, mature and proliferate producing vertical structures within the growth plate. When chondrocytes increase in size, they become hypertrophic and do not synthesize type II collagen. Instead, they start to express type X collagen, which has regulatory effect on calcium deposition into the matrix. Alkaline phosphatase is also synthesized within this transition region between bone and cartilage. Furthermore, hypertrophic chondrocytes induce production of vascular endothelial growth factor (VEGF) for the formation of blood vessels under the growth plate. Eventually, degenerated chondrocytes die by apoptosis and are engulfed by phagocytosis. The remaining empty space is replaced by osteoclasts through a process called endochondral ossification, by which hypertrophic cartilage is replaced by bone (Figure 2.1) [11-14].

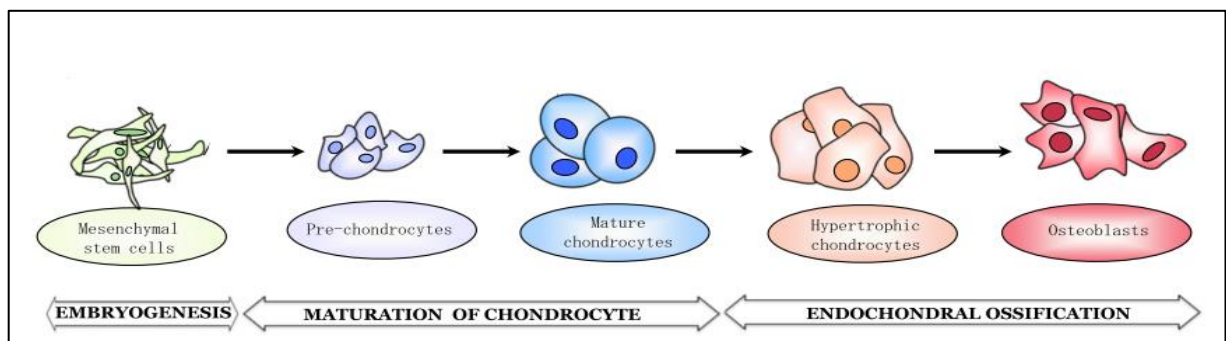


Figure 2.1. Schematic representation of chondrocytes at different stages of chondrogenesis[14]

The 65 to 80 percent of the articular cartilage matrix is composed of water, and the remaining wet weight constitutes collagen, proteoglycans, glycoproteins and other cartilage specific proteins [15]. The constant diffusion and exchange rate of water through the cartilage matrix accompanied by joint compression provide nutrient transport to chondrocytes. Proteoglycans represent approximately 8 percent of the total weight of the cartilage. The major proteoglycan

found in the cartilage tissue is aggrecan. Proteoglycans consist of a high molecular weight protein core that covalently attaches to the glucosaminoglycans (GAGs). Hyaluronic acid, chondroitin-sulfate, keratan-sulfate, and dermatan-sulfate are the most abundant GAGs in the structure of cartilage. The negatively charged GAGs are responsible for the high water content of the cartilage, since they attract water molecules and eventually result in increasing their volume [7,15,16].

Collagen accounts for ~50 percent of the dry weight (~10 -11 percent of total weight) of the ECM. Type II collagen is the most abundant structural element of the cartilage tissue. It has triple helix conformation composed of three $\alpha 1$ (II) polypeptide chains. The $\alpha 1$ monomers are synthesized as propeptides having large N- and C- terminal regions that are needed for chondrocyte assembly. Furthermore, this distinctive structure of collagen makes it insoluble and resistant to most degradation enzymes. However, matrix metalloproteases (MMPs) are capable of targeting $\alpha 1$ helix in the structure of type II collagen. Beside type II collagen, collagen types IX, X, XI, VI and a small amount of type I collagen exist in the ECM of cartilage tissue [7,16,17,18].

Articular cartilage has four different regions; superficial (tangential) zone, transitional (middle) zone, deep (radial) zone, and calcified zone (Figure 2.2). The superficial zone corresponds to 10–20 percent of the cartilage thickness, and is composed of dense collagen fibrils parallel to the surface. It contains high amounts of decorin and lubricin, and low amount of aggrecan. The low proteoglycan concentration causes higher permeability than the other regions of cartilage. In addition, the collagen architecture is optimal for bearing shear forces. The chondrocytes in this zone are flattened and control appositional growth. The transitional zone (40–60% of the cartilage thickness) is composed of thicker collagen fibrils, compared to other regions, with a random organization. Chondrocytes in this layer appear rounded and produce large amounts of type II collagen and proteoglycan. On the contrary, the cell density decreases within this zone. The 20–50% of the cross sectional length corresponds to deep zone along with the calcified zone. The deep zone consists of thickest collagen fibers perpendicular to the calcified zone and the subchondral bone. Chondrocytes located in this zone express collagen type X, a hypertrophic marker, and less type II collagen. They become larger, and appear elongated in the same

direction of collagen fibers. The tidemark region separates deep and calcified zones from each other. The calcified zone consists of hydroxyapatite and they function as a transition region between cartilage and bone [19-21].

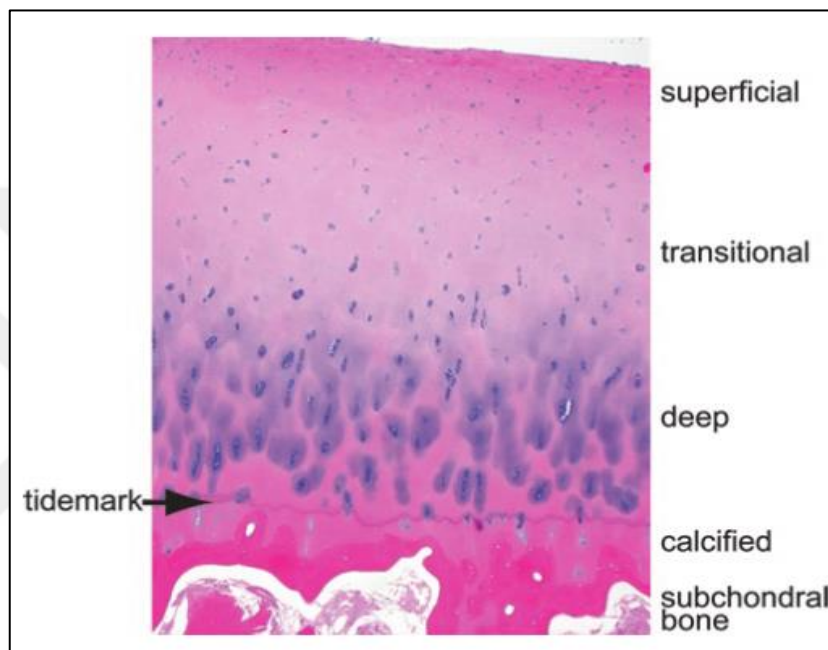


Figure 2.2. Microarchitecture of articular cartilage [7]

Articular cartilage is exposed to different types of mechanical stimuli like hydrostatic pressure, shear and compressive loads, under physiological conditions. The composition and structural arrangement of the ECM provides mechanical strength to the articular cartilage. While the type II collagen network is responsible for the tensile strength of the cartilage, the water repelling proteoglycans are responsible for cartilage resistance to compressive forces [22]. Mechanical loading has an influence on MSC differentiation towards chondrocytes during articular cartilage development. It also helps chondrocytes for regulating and maintaining their phenotypes. Furthermore, mechanical loads function as a stimulatory factor for the production of ECM molecules such as collagens, GAGs and proteins, which turn into a feedback mechanism between loading and matrix synthesis [23,24].

The fibrocartilage is another type of cartilage that is rich in dense collagen fibers parallel to each other in several layers. It contains type I collagen, in addition to the type II collagen. It develops at sites such as articular discs, symphysis joints, lining of bony grooves, where the tissues are subject to shear and compressive forces. The elastic cartilage matrix is composed of dense elastic fiber networks. It is mainly found in non-load bearing body parts, such as larynx, ears and nose [6,8].

2.2. CARTILAGE TISSUE ENGINEERING

Treatment of damaged cartilage, caused by injuries or diseases, is one of the most challenging issues in orthopedics due to the limited self-repair capacity of the tissue. Cartilage diseases can be resulted from external injuries, genetic mutations that encode cartilage specific ECM molecules or regulatory proteins (chondrodysplasias, achondrogenesis type II etc.), inflammations (relapsing polychondritis, rheumatoid arthritis etc.) congenital disorders (dwarfism, achondroplasia etc.), degenerative disorders (osteoarthritis) or cartilaginous tumors [25]. Current treatment methods involve bone marrow stimulation techniques such as drilling, abrasion arthroplasty, and microfracture that aim to trigger mesenchymal stem cell migration to the chondral and provide blood supply by opening channels through the subchondral bone plate [26]. Total joint arthroplasty, mosaicplasty, and autologous chondrocyte implantation are the other techniques for repairing cartilage. However, all these methods possess some drawbacks and limitations. The newly formed tissue cannot compensate the mechanical and functional characteristics of the native cartilage, and the surgical techniques do not preclude further degeneration of the damaged tissue [27]. Furthermore, the methods may also cause donor side morbidity, fibro cartilage formation, and dedifferentiation of chondrocytes after implantation, and these consequences may jeopardize healthy tissues [28,29]. When all the adversities are taken into account, cartilage tissue engineering becomes an alternative solution to surpass the limitations of conventional orthopaedic treatments.

Tissue engineering is a multidisciplinary field that combines the knowledge of engineering and life sciences in order to construct functional and feasible substitutes that repair, preserve, or

improve the damaged or diseased tissues [30,31]. The strategy involves cells, scaffolds, and stimulating factors in variable combinations or solitarily. The ideal scaffold should be biocompatible, biodegradable, non-cytotoxic, and permeable enough for the transportation of nutrients, metabolic wastes, and bioactive molecules, mechanically stable, reproducible and able to support the cells as they synthesize new matrix components [32]. It is important to design an optimal scaffold for the construction of cartilage tissue since chondrocytes cannot preserve their chondrogenic phenotype when they are expanded in a monolayer culture. There have been various materials used for cartilage tissue engineering up to present day. These biomaterials can be categorized into two groups: synthetic and natural biomaterials. They can be interchangeably used for cartilage tissue engineering with respect to their structural properties. In addition to cell-scaffold interaction, stimulating factors are also needed for inducing differentiation, proliferation and metabolic activity of the cells [33].

2.2.1. Cell Source

The extracellular matrices are synthesized by the cells, and responsible for the mechanical properties and the shape of a tissue. For this reason, the selection of the ideal cell source for cartilage tissue engineering is very substantial matter. Cells should be isolated easily, have expansion capacity, and can be cultured to produce molecules specific to cartilage tissue in order to be used in cartilage tissue engineering [34]. Although the search for identifying the optimal cell source is still in progress, scientists have investigated stem cells, genetically modified cells, chondrocytes, and fibroblast for the repair of cartilage [29, 35]

Stem cells are unspecialized cells that are able to differentiate into more specialized cell types. They have the capability to divide themselves continually to produce more stem cells or differentiate into various types of cells. Embryonic stem cells, fetal derived stem cells and adult stem cells are the most studied stem cells in the field of tissue engineering and regenerative medicine.

Embryonic stem cells (ESCs) are self-renewing and pluripotent cells that are able to differentiate into all cell types arisen from ectoderm, endoderm, and mesoderm [36]. In previous studies,

ESCs were differentiated to chondrocytes using growth factors [37,38] and different chemical supplements [39], co-cultured with chondrocytes to improve the chondrogenic differentiation capacity [40], and induced to be converted to mesenchymal stem cells [41] or progenitor cells [42] in order to be used in cartilage repair strategies. Although ESCs appear to be promising candidates for cartilage tissue engineering applications, there are still ongoing debates about the ethical and social concerns in ESC research since they are isolated from the inner cell mass of blastocysts [43]. The risk of immune-rejection and the possibility of teratoma formation are the other difficulties encountered during clinical application studies of ESCs [44].

Unlike ESCs, mesenchymal stem cells (MSCs) reside in each tissue of the body, and can only be activated by trauma, disease or aging [29]. MSCs are self-renewable, multipotent cells that are capable to differentiate into chondrogenic, osteogenic, adipogenic and myogenic lineages. They can be isolated from bone marrow [45,46], peripheral blood [47], muscle [48], adipose tissue [49], umbilical cord (Wharton's jelly) [50], pancreas, skin, dental pulp, and neuronal system [43, 51].

In vivo chondrogenic differentiation process of MSCs begins with the molecular signals emitted by surrounding tissue cells. Those signals comprise the stimulation of the stem cell surface receptors, cytokines and growth factors. The signals can also be stimulated through ECM proteins such as collagens, glycosaminoglycans and proteoglycans or through the neighbouring cell (e.g. chondrocytes) interactions. Therefore, the current approaches of *in vitro* chondrogenic differentiation of MSCs are based on mimicking the chondrogenesis, homeostatic mechanisms and functions of native cartilage [51].

Bone marrow is a widely used cell source for the isolation of mesenchymal stem cells. Bone marrow mesenchymal stem cells (BM-MSCs) can differentiate into chondrocytes under various culture conditions. The most common strategy involves the stimulation with transforming growth factor beta (TGF- β) [52,53] in a 3D culture environment, such as cell pellets and scaffolds, or through the combinations of multiple growth factors [54]. Numerous scaffolds have been used for chondrogenesis of BM-MSCs including hyaluronic acid, alginate, silk, poly (ethylene glycol) (PEG), polycaprolactone (PCL), collagen, fibrin, chitosan and others [28].

However, the level of chondrogenesis highly depends on the choice of scaffold material, for this reason researchers combined various materials to improve chondrogenic development. For example, Ma *et al.* prepared fibrin/alginate hydrogels with different concentrations to show the variations in chondrogenesis of BM-MSCs that were seeded in these hydrogels [55]. Moreover, polyurethane constructions [56], collagen [57] and ECM [58] mimetic matrices, and silk fibroin/gelatin–chondroitin sulfate-hyaluronic acid blend [59] scaffolds were also used in *in vitro* chondrogenesis of BM-MSCs.

Adipose-derived mesenchymal stem cells (ADMSCs) can be isolated from adipose tissue by lipoaspiration procedure. The isolation process is relatively easy and vast amount of cells can be harvested from several adipose tissues [60]. The ADMSCs can be cultured *in vitro* for a long period of time, since they have low senescence rates up to 15 passages [61]. When compared to BM-MSC in terms of multilineage differentiation capacity and growth kinetics, there was no significant difference found between two mesenchymal stem cells [62]. On the other hand, there are some other reports available supporting the exact opposite of this situation. For instance, Xie *et al.* showed that BM-MSCs had higher proliferation rate, and higher expression of cartilage-specific genes and proteins than ADMSCs [63]. Additionally, Puetzer *et al.* indicated the difference of cell populations obtained from bone marrow and adipose tissue by investigating the previous studies and concluded that they required different stimulative supplements to reach ideal chondrogenic differentiation [64].

Induced pluripotent stem cells (IPSCs) are produced from adult somatic cells. They were genetically reprogrammed to an embryonic stem cell-like state for the first time by Takashi and Yamanaka [65]. Oct3/4, SOX2, Klf4 and c-Myc were the four transcription factors that came into prominence among 24 selected gene candidates for inducing pluripotency and maintaining ESC-specific properties. These transcription factors were introduced into mouse fibroblast cells by retroviral transduction and resulted in pluripotent clones [65]. IPSCs do not have ethical concerns like ESCs and they have high proliferative activity. They can be expanded virtually indefinite time without undergoing senescence. Chondrogenesis using IPSCs has been achieved with various methods. For example, after the chondrogenic differentiation, IPSC-derived chondrocytes were seeded in/on a scaffold using tissue engineering methods to create cartilage-

like tissue *in vitro*. Alternatively, the resultant chondrocytes can directly be transplanted into the damaged or diseased areas of cartilage tissue [66,67]. Yamashita *et al.* formed cartilaginous particles from iPSCs without scaffolds to create a viable cell source for the treatment of cartilage defects [68]. Notwithstanding the promising studies done with iPSCs, there are some impediments that need to be overcome before translating iPSC research to clinical trials such as risk of teratoma formation, chondrogenic impact and FDA validation [69].

The most obvious cells used in cartilage tissue engineering are chondrocytes. The cartilaginous matrix is synthesized by these cells and they are the main cells of healthy cartilage. Chondrocytes are generally obtained from the patients' cartilage and expanded in the laboratory. Although chondrocytes have been extensively used in studies regarding cartilage regeneration so far, they may cause some significant problems such as donor site morbidity, insufficient cell resources and the instability in monolayer culture [29]. Dedifferentiation problems can be surmounted by the cultivation of chondrocytes in proper 3D-environment. Furthermore, allogenic and cryopreserved chondrocytes might be alternative sources of chondrocytes for cartilage reconstruction [70].

Moreover, co-cultures of various cell types have been investigating to enhance the chondrogenic and proliferative potentials of the engineered cartilage [71-73].

2.2.2. Scaffolds

The search for the ideal material for the construction of cartilage tissue has been continuing for many years. Until now, a wide variety of scaffolding materials have been studied for *in vitro* and *in vivo* cartilage regeneration. The first necessity of the optimal scaffold is biocompatibility. Scaffolds should be in contact with the living systems without triggering an immune response or inflammatory reactions [28,74]. They should also be non-toxic, non-carcinogenic, and non-irritating. Secondly, the scaffolds should be biodegradable with the proper rate of degradation that is proportional to the production of new cartilaginous tissue. The biodegradation mechanisms of materials are generally categorized as surface erosion and bulk erosion. While the surface erosion occurs at constant velocity at any time during the process, bulk erosion

occurs spontaneously [75]. The water uptake of hydrophilic polymers may lead to bulk erosion. Hydrolytic degradation process requires water to breakdown polymers into monomers. The degradation rate changes with the amount of covalent bonds or non-hydrolysable bonds in the backbone. When those types of bonds are highly present in the polymer, the degradation rate decreases. This process can also be accelerated by the acids, bases or salts. Most of the synthetic biodegradable materials have tendency towards releasing acidic by-products that cause a reduction in the pH. The alteration in the pH stimulates the autocatalytic degradation process. On the other hand, hydrophobic polymers usually undergo surface degradation, which is more advantageous since the degradation rates can be controlled and the structural characteristics of the polymers can be preserved for longer period of time [76].

Polymeric materials may also undergo enzymatic and oxidative degradations. In a biological environment, both natural and synthetic polymers can be degraded by the enzymes such as proteases, esterases, glycosidases, and phosphatases. The enzymatic degradation begins with the diffusion of the enzymes from the circumambient solution into the material. After diffusion, enzymes adhere to the material and catalyze the hydrolysis reaction that ultimately leads breakdown of the material. In oxidative degradation, materials should directly contact with body tissues or fluids. The degradation process can be catalyzed by reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) [77]. In this regard, it is important to consider the biocompatibility of the degradation products of the materials within living systems as well.

Porosity, cell-polymer interactions and mechanical compatibility are the other essential features for cartilage tissue engineering scaffolds. The polymers should be porous enough to allow the diffusion of molecules, nutrients, wastes and cell migration. They should accommodate to native cartilage by integrating themselves to the host tissue. The cell proliferation, differentiation and the production of ECM components of the cartilage tissue should be promoted by the scaffolds. Furthermore, the method for cell seeding (statically or dynamically) and the cell seeding density have to be considered in order to ensure sufficient cell-cell interactions. Higher seeding densities can induce interactions of cells between each other and stimulate ECM synthesis. In addition, the dynamic cell seeding can ameliorate the cellular distribution, which in turn provides the

formation of more uniform tissue. Moreover, the newly formed tissue should be able to fill the defect with any size and shape [28, 33,34,78,79].

Lastly, the scaffolds should be mechanically compatible with the healthy cartilage tissue. Upon implantation, the constructs must bear most of the physiological load of the host tissue. During tissue regeneration, the increasing levels of mechanical loadings can stimulate tissue growth and act like stimulating factors for the synthesis of the suitable cartilaginous ECM. Besides, they have effects on *in vitro* and *in vivo* MSC differentiation towards chondrocytes [23, 80]

Overall, designing an ideal scaffold for the cartilage tissue engineering is a troublesome process, therefore one should consider all the advantages and disadvantages of the scaffolding material, and make a decision regarding the necessities.

2.2.2.1. Natural Polymeric Scaffolds

Protein based (e.g. silk, collagen, fibrin etc.) or polysaccharide based (e.g. chitin/chitosan, hyaluronic acid, cellulose, alginate, and gelatin, etc.) polymers are widely used natural scaffolding materials for the cartilage tissue regeneration. Natural polymers are favorable due to economic and environmental aspects. They have low manufacturing and disposal costs, low toxicity and high renewability. In addition, these polymers can interact with cells with their surface receptors and direct biological signaling, and cell adhesion and function. They also contribute to cell responsive degradation and re-modelling. However, naturally occurring polymers might have low mechanical, thermal and chemical instability, and a risk of immune rejection and disease transmission. Yet these problems can be overcome with the physical and chemical modifications of the natural polymers [34,76,81].

Silk is a fibrous protein that can be obtained from various spiders and insects. The most commonly used silk is the cocoon silk of the *Bombyxmori* silkworms. Silkworm silk is composed of two proteins: fibroin and sericin. The filaments of hydrophobic fibroin are surrounded and conjoined by glue-like hydrophilic sericin proteins. However, in order to be used in biomedical applications, the silk fibroin is needed to be purified since residual sericin

may trigger an immune response. Silk fibroin is highly preferable due to its higher strength and flexibility, thermal stability, slow degradation, water-oxygen permeability, and genetic and chemical tailorability [81-83]. Therefore, silk fibroin based scaffolds in the forms of hydrogels [84], electrospun fibers [85], and films [86] have been extensively used in cartilage tissue engineering with MSCs and chondrocytes.

Collagen is the most abundant protein found in the ECM of the various tissues such as bone, cartilage, skin, and cornea. It is the major component of the connective tissue and about 25-35 percent of all proteins in the body consists of collagen. Low antigenicity, biocompatibility and good cell attachment make collagen a good candidate for cartilage tissue engineering applications, but because of the low biomechanical stiffness and fast biodegradation properties, collagen is needed to be modified before using as a scaffold [28,87]

Fibrin is a protein produced temporarily during tissue injuries. It is formed by the polymerization of fibrinogen monomers that are present in the blood, which coagulates to create fibrin clots upon injury [88]. Fibrin forms a transient matrix subsequent to vascular injury that in turn triggers cells to migrate through the damaged tissue. The increasing cellular activity accelerates tissue regeneration, which makes fibrin favorable biomaterial [89]. Furthermore, fibrin possesses fast polymerization kinetics and it is easy to manipulate and isolate from blood plasma. It has been used as a scaffold in many *in vitro* and *in vivo* cartilage tissue engineering studies alone or in combination with other biomaterials so far [90-93].

One of the most known polysaccharide-based natural polymers is hyaluronic acid (hyaluronan, HA). It is a non-sulfated glycosaminoglycan discovered by Karl Meyer and John Palmer in 1934 [94]. They described a method for the isolation of a glycosaminoglycan from the vitreous humour of bovine eyes. HA is composed of two sugars, D-glucuronic acid and N-acetyl-D-glucosamine, polymerized into large macromolecules containing more than 30000 uniformly recurring units and its molecular structure is highly conserved between mammalian species such as the rat, rabbit and human (Figure 2.3).

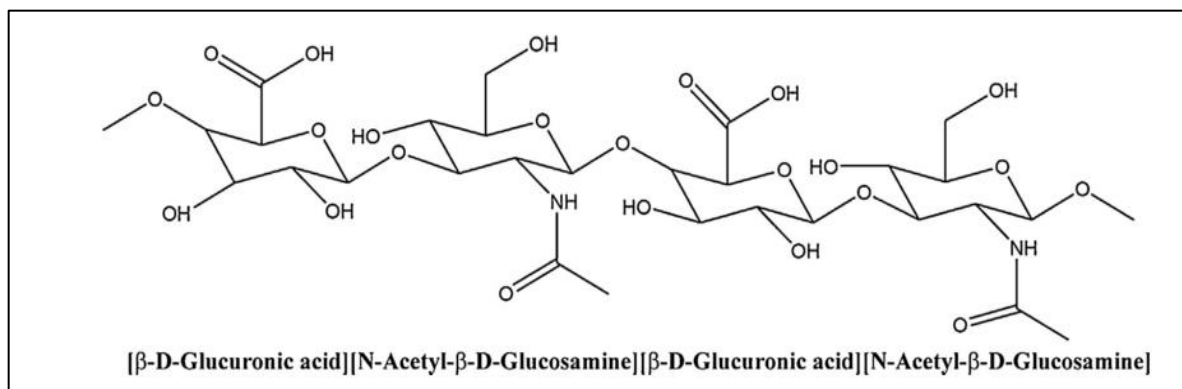


Figure 2.3. Chemical structure of hyaluronic acid (HA) [95]

Up to present, HA has been utilized in different medical applications as a filling material, drug delivery agent, scaffold for tissue regeneration and a protector of embryo during *in vitro* fertilization. Like all other natural polymeric scaffolds, HA is also biodegradable, biocompatible and bioresorbable material. It is the prominent intracellular component of the connective tissue, which has a significant role in lubrication, cell growth and differentiation. It can be recognized by some cell surface receptors such as CD44, the receptor for hyaluronan mediated motility (RHAMM), and some Toll-like receptors [95,96]. Cell-cell and cell-matrix adhesion are affected by the binding of HA to its major receptor CD44. In addition, HA scaffolds can bind to chondrocytes over CD44 and can induce chondrogenic gene expressions of BMSCs and ADMSCs [97, 98]. RHAMM is generally localized inside the cell. Upon stimulation by wounding and cytokines [99], its expression increases in both intracellular and extracellular space, which is needed for the cytoskeletal rearrangement and scaffold mediated cell locomotion [100].

Furthermore, there is a theory which defends the positive effects of HA on the enhancement of immunosuppressive properties of MSC's [101]. It is entitled as 'Signaling Theory' and originated from the observations that HA displays different responses on cell behavior with contingency on varying molecular size of HA. It is the known fact that low molecular weight HA (LMWHA) regulates the inflammatory responses and acts as an intrinsic danger signal through Toll-like receptor 2 (TLR-2) signaling on macrophages and dendritic cells. On the

contrary, high molecular weight HA (HMWHA) hinders TLR-2 signaling and induces peripheral blood monocytes for the expression of growth factors and matrix components instead of pro-inflammatory components [102]. According to these findings, HMWHA provides immunotolerance by producing a stimulatory signal.

HA is highly soluble in water and when exposed to water, it becomes a gel. The high solubility of HA in water can be an impediment to the production of polymers for tissue engineering because chemical and structural stabilization are required for scaffolding. Moreover, it can be enzymatically degraded by hyaluronidases. In order to overcome this limitation, functional domains can be introduced to the functional groups (carboxyls, hydroxyls/acetyls) found on the backbone of HA, or functional groups can be crosslinked with some crosslinkers such as carbodiimide-, polyvalent hydrazide-, disulfide-, divinyl sulfone-, biotin, ceramide-, formaldehyde- and glycidyl methacrylate-HA conjugates (photocrosslinker). In this way, the stability of the HA polymer can be increased [95,103].

Chitin is the second most abundant polymer in nature and found in the structure of exoskeleton of arthropods or in the cell walls of fungi and yeast. The polymer is also produced by some animals and plants to provide strength and reinforcement. Chitosan is originated from chitin, obtained by partial deacetylation of chitin in basic ($\text{pH} > 7$) solutions or by enzymatic hydrolysis in the existence of chitin deacetylase. Chitin and chitosan are non-toxic, tailorable, antibacterial, biocompatible and degradable (by enzymes) polymeric biomaterials used in tissue engineering as well as biomedical applications such as wound healing, drug and gene delivery. Chitosan has been studied in the forms of hydrogels, nanofibers, beads, sponges, macro/nanoparticles for many years [104-106]. Because of the aforementioned properties, chitosan was used and will continue to be used as a scaffold in cartilage tissue engineering [107-111].

Cellulose is a linear polysaccharide consisting of the repeating units of glucose ($\text{C}_6\text{H}_{10}\text{O}_5$) monomers, ranging from 500-5000. It can be degraded by enzymes and its water solubility depends on the number of monomers in the chain. Since it is easy to process, cellulose can be formed into a wide range of shapes [112]. In recent years, engineered form of cellulose has been used as a scaffold for many cartilage tissue engineering applications [113-116]. Bacterial

cellulose (BC) is usually produced by *Gluconacetobacter xylinus* (*Acetobacter xylinum*) via static suspension culture in a liquid medium. It is more advantageous than the cellulose derived from plant source due to its higher water holding capacity and higher permeability [117].

Alginate is another linear polymer that contains 1-4-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) units coalesced in an irregular arrangement of varying ratios of GG, MG and MM blocks. Alginate gels can be produced in aqueous medium containing bivalent cations (e.g. Ca_2^+) in a reversible manner. They can be dissolved rapidly using a calcium chelating agent [118-120]. Therefore, it is easy to utilize alginate as an injectable cell delivery system or for encapsulation. For this purpose, Debnath *et al.*, encapsulated human ADMSCs in alginate microspheres in order to induce chondrogenesis [121]. Balakrishnan *et al.*, designed an injectable alginate/gelatin hydrogel for cartilage regeneration [122]. In another study, a 3D highly organized, porous alginate scaffold was prepared with a microfluidic device. Subsequently, chondrocytes were seeded on these scaffolds, and maintained their phenotype while proliferating [123].

Gelatin is produced by the partial hydrolysis of collagen. It is economic, highly available, biocompatible and biodegradable natural material. The degradation products of gelatin are harmless, but they have very low antigenicity. In order to avoid the potential transmission of infectious disease, human recombinant gelatin can be produced with different genetic engineering approaches. Apart from these, the presence of the cell-recognition motifs (Arg-Gly-Asp (RGD)) in the structure of gelatin, improves the biological performance of the polymer [124]. Furthermore, United States Food and Drug Administration has recognized gelatin as a GRAS (Generally Regarded as Safe) material, so that it is convenient to use gelatin in cartilage tissue engineering applications as well as many others [124].

There are also other types of natural materials that can be promising candidates for the construction of cartilage tissue engineering scaffolds. LBG and XG are the two of these materials which have been used in many different fields except cartilage tissue engineering.

Locust Bean Gum, Xanthan Gum and Mastic Gum

LBG is a galactomannan, a member of the family of polysaccharides, consisting of a (1–4)-linked β -D-mannopyranose backbone with side chains of (1–6)-linked α -D-galactopyranose attached to every fourth or fifth mannose (Figure 2.4). Galactomannans are storage polysaccharides found in the structures of different plant seeds and supply energy to the endosperm for the germination process. LBG is isolated from the seed endosperm of the carob tree (*Ceratonia siliqua*) [125]. Turkey is the fifth country in the production of carob seeds worldwide [126]. Carob production in Turkey was 16256 tonnes in 2019 [127].

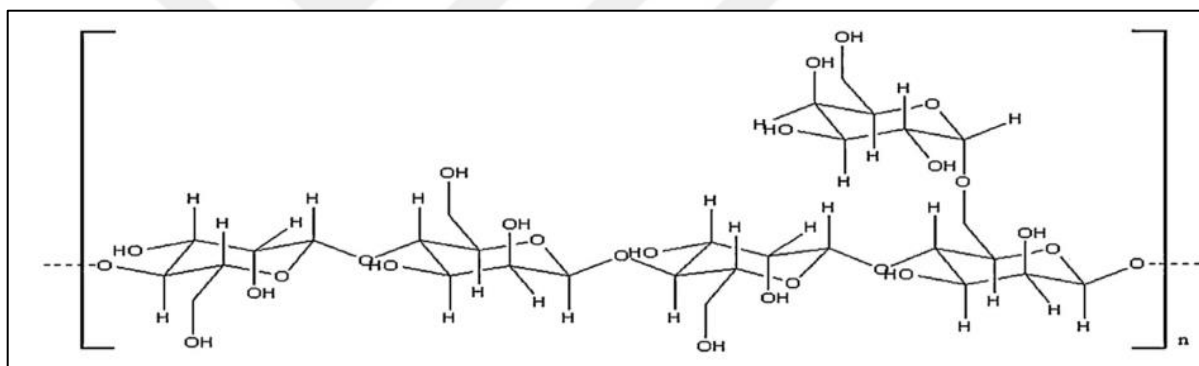


Figure 2.4. The chemical structure of LBG [125]

Carob seeds are commercially processed through the steps of endosperm separation after dehusking and germ splitting, grinding and sifting of the endosperm into fine particle-sized flour, and precipitation-purification. LBG contains proteins, lipids, ashes etc. aside from galactomannan (Table 2.1) [128]; therefore, it is important to remove impurities via enzymatic or alkaline hydrolysis, ethanol or isopropanol precipitation, and purification using methanol, copper or barium [129].

Table 2.1. Composition of LBG [128]

Constituent	Proportion (%)
Galactomannan	80.0-85.0
Moisture	10.0-12.0
Protein content	5.0-6.0
Fat content	0.5-0.9
Crude Fiber	0.8-1.0
Ash	0.5-1.0

Galactomannans differ from each other by their mannose to galactose ratios (M/G). The different ratios are arisen from the origin of the plant species or their growth conditions. This ratio has an influence on the solubility of galactomannans that increases with the higher the substitution of galactose. The interchain association and crystallization of galactomannans are disturbed by steric effects of the galactose side groups, so galactomannans become readily soluble in water. In this context, higher percentage of galactose provides good solubility in cold water. For this reason, LBG (which has 3.9-4.0:1.0 M/G ratio) demonstrates limited solubility in water [125,130]. However, it is indicated that LBG reaches 50 percent solubility after 1 hour stirring, and a maximum solubilization (70-85 percent) can be achieved by 30 min stirring at 80 °C [131].

Galactomannans form colloidal dispersion when hydrated in water. The rate of hydration of LBG depends on the quality of the commercial product, which is based on the particle size, gum content and the amount of impurities. When galactomannans are in a solution, they form rod-like shapes that gyrate through the solvent, and eventually collide with each other and with the aggregates of the solvent molecules to generate viscous solutions [130]. Since LBG requires heating for its full hydration, hot water is needed to obtain viscous LBG solution. Besides, the thickening capacity of LBG relies on some other factors such as polymer concentration, shear rate, molecular weight distribution and solubilization procedures. It is capable to form very viscous solutions at relatively low concentrations. Because of the neutral structure of LBG, these viscous solutions are nearly unaffected by pH, or salts [128].

LBG is a biocompatible, bioresorbable, renewable and an edible natural polymer. It was categorized under GRAS materials by FDA. LBG can be degraded by the enzymes, β -mannanase, β -mannosidase and α -galactosidase, found in the body. Figure 2.5 shows the schematic diagram of the enzymatic degradation of LBG [132-134]. The degradation products can be easily eliminated from the body.

LBG produces a weak gel under normal circumstances. However, it displays a remarkable capacity to form synergistic interactions with other polysaccharides. The presence of numerous -OH groups in the molecular structure of LBG, increases its tendency to produce gels with other polysaccharides such as XG [128,130].

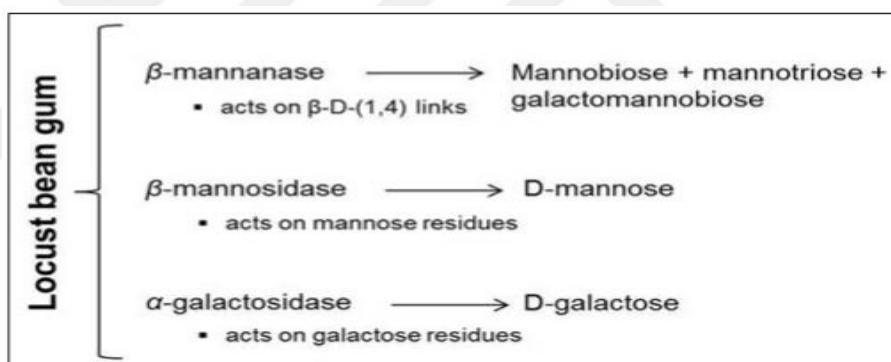


Figure 2.5. The schematic diagram of the enzymatic degradation of LBG [130]

Xanthan gum (XG) is an exo-polysaccharide discovered at the laboratories of United States Department of Agriculture in 1950s [135]. It is a polyanionic polymer produced by gram-negative plant pathogenic bacterium, *Xanthomonas campestris*. The xanthan backbone is formed by sequential additions of D-glucose-1-phosphate and D-glucose, D-mannose and D-glucuronic acid, O-acetyl groups to the internal mannose and finally pyruvate to the terminal mannose. Specific enzymes and substrates are required at each step for completion of reactions. If any of these substrates or enzymes is absent, the polymerization reaction cannot be completed [136].

XG contains two (1-4)- β -D-glucose units on the main chain and a terminal trisaccharide side chain (β -D-mannose, β -D-glucuronic acid and β -D-mannose) on every second C-3. In addition, XG also contains a pyruvic acid residue linked to the side chains and O-acetyl group linked to the main chain (Figure 2.6) [136,137].

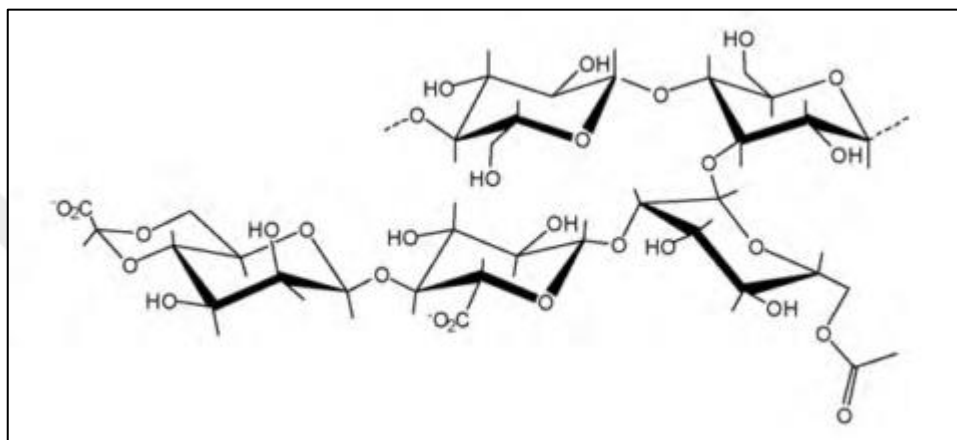


Figure 2.6. The chemical structure of XG [138]

XG is a stable material over wide temperatures and has good shear-thinning behavior. It is biocompatible, tailorable, reproducible, non-toxic material, which has high water solubility and limited electrical conductivity. Furthermore, it has been approved by FDA to be used as a food additive without any quantity restrictions. Besides food industry, XG has broad range of applications from petroleum industry to pharmaceuticals, cosmetics and personal care products [136-138].

Because of the aforementioned characteristics, XG has been started to get used in tissue engineering applications recently. XG is widely used with LBG in the form of hydrogel in many different fields. It cannot form gel naturally at any concentration, however, when it is mixed with β -(1,4) linked polysaccharides, like LBG, strong, and thermos-reversible gels can be produced [140]. The synergistic interactions between LBG and XG were discovered by Rocks [141] for the first time, and then studied by many other researchers until now [140, 142-147]. Although, this synergistic effect has been studied extremely, the exact mechanism remains an

enigma. However, several models have been proposed. The first model involves cooperative interactions between ordered segments of rod-like XG and unsubstituted parts of LBG backbone. The second model suggested a lock-key mode of interactions in which xanthan side chain associates with unsubstituted zones of the LBG backbone. The last model promotes the idea of denatured Xanthan is needed for intermolecular interactions of Xanthan and LBG and thus gelation [148].

LBG and XG gelation process depends on the weight ratio between two polysaccharides and the temperature during preparation. For example, Sandolo *et al.* observed that 1:1 LBG/XG ratio produced a gel under any conditions, and the ratio of 1:3 led weak gel formation at 75°C, while the ratio of 1:9 could not produce a proper gel [138]. Similarly, Renou *et al.* investigated the effect of the thermal treatment of XG/LBG mixtures on their interactions and revealed that rheological properties were strongly improved after heating XG in the presence of LBG. They explained that such improvement can only be associated with the increased binding of disordered XG regions to smooth regions of LBG. Furthermore, it was also demonstrated that XG should have at least 35 percent pyruvate groups to interact with LBG, and the interactions can occur regardless of the amount of acetate content with varying intensity [148].

Hydrogels are polymeric networks fabricated through physical and chemical cross-linking. They are characterized by high water holding capacity which is similar to native cartilage tissue [34]. Cryogels (macro-porous hydrogels) can be obtained from LBG and XG that allow nutrient transportation and removal of metabolic wastes. Pore size is important for the permeability of the material and the permeability of a scaffold increases with increasing pore size [149]. When cartilage is under physiological load, fluid flows out of the solid matrix composed of proteoglycans, collagen, cells, and lipids, and decreases the volume of the whole cartilage, thus bears the forces applied by the load. Therefore, arranging the rate of permeability is a substantial phenomenon for satisfying the mechanical requirements of cartilage. On the other hand, proteoglycans are the main molecules that provide the mechanical strength to cartilage. When cartilage is compressed, the negatively charged sites on proteoglycans are pushed closer together, which increases their mutual repulsive force and adds to the compressive stiffness of the cartilage [150]. Considering this information, polyanionic nature of XG can promote

compressive stiffness of the LBG/XG scaffold system as it is in the native cartilage tissue. Furthermore, interconnected macro-porous network promotes attachment, proliferation and migration of the chondrocytes, and cartilage-specific matrix production within the scaffolds. Chondrocytes can also maintain their phenotypes within these cryogels [151,152].

In the light of this information, the special synergistic behavior, tunability and the biocompatibility of the LBG-XG make these two polysaccharides promising natural scaffolding materials for cartilage tissue engineering.

Mastic gum (MG) is a natural resin obtained from the stem and leaves of *Pistacia lentiscus* (Family *Anacardiaceae*) evergreen shrub or trees which are grown in Mediterranean region [153]. It has been used as culinary material, and for therapeutic purposes in Mediterranean and Middle Eastern countries for centuries so far [154]. Mastic tree was originally cultivated in southern part of the island of Chios (Chios Mastic Gum (CMG)), later, they were widely distributed to Mediterranean regions, including Turkey (Figure 2.7). *Pistacia* species are grown in Aegean and Mediterranean parts of Turkey, within forests, alone or with other species such as olive, carob, pine and terebinth trees, or in stony seaside areas [155,156]. MG is composed of many different compounds and produced in the form of pale-yellow drops obtained from the insicions made on the trunk of the tree or branches of shrub. Mastic resin is the major compound of MG, which consists of triterpenes (approximately 65-70 percent), essential oil, volatile compounds, and mastic water [157]. Terpenes are large molecules categorized according to the number of carbons as well as presence, position, and number of hydroxyls, ketone or acidic functional groups in their molecular structure [158]. Triterpenes consist of dammarane precursor and 30 carbon atoms, in which hydroxyl groups present nearly always in position 3 [159]. They are a large family of natural compounds present in various living organisms, especially in plants. Triterpenes have anticarcinogenic potential since they possess antiproliferative and antineoplastic properties [160]. The MG has acidic and neutral parts (Figure 2.8). While oleanonic, 24Z-isomasticadienonic, 24Z-isomasticadienolic, 24Z masticadienonic acid, 24-Z masticadienolic, and moronic acids are the acidic fractions, tirucallol, 28-nor-olean-17(18)-en-3-one, oleanonic aldehyde, dammaradienone constitute the neutral fractions of MG [161-163]. The essential oil of MG is composed of alpha-pinene, beta-pinene, beta-myrcene, carvacrol, camphene,

limonene, β -caryophyllene, verbenone, α -terpineol, and linalool. The essential oil constituents differ among mastic tree species, plant parts that are used during extraction, cultivated geographical area, and extraction methods [161]. The mastic oil has been used as an anti-inflammatory, antimicrobial, and antileukemic agents, as well as in the cosmetics and food industries [164]. The last component of MG is the water produced during the steam distillation process of mastic resin. The mastic water has proven to be antimicrobial [165] and antigenotoxic [166] in the literature. Although CMG is the most studied type of MG in the literature, *Tabanca et al.*, characterized the essential oil obtained from the Mastic trees cultivated in Çeşme–Uzunköy, Çeşme–Germiyan and Mordoğan in İzmir, Turkey. It was found that α -pinene, β -pinene, and myrcene ratios in essential oils varied regarding the cultivated areas of mastic trees [167].



Figure 2.7. The image of *Pistacia lentiscus* tree [168]

The MG was first used by Egyptians in 7th century BC in embalming of mummies and as a glue. Afterwards, it has been used as phyto-therapeutic agent, cleaning product for the oral cavity,

skin caring agent, hardening and shining agent in paints so far [157]. In addition, it was used in cosmetic products, chewing gums, toothpaste, liquors, and lotions for decades. Furthermore, the positive effects of MG have been shown for gastrointestinal disorders like ulcers [169], *Helicobacter pylori* infection [170,171], and pancreatic cancer [154]. The MG was also utilized as matrix forming material for cell encapsulation [172] and sustained drug release [173]. The anticarcinogenic, cardioprotective, antibacterial, and antioxidant properties [157] of MG make it desirable for many different fields of research.

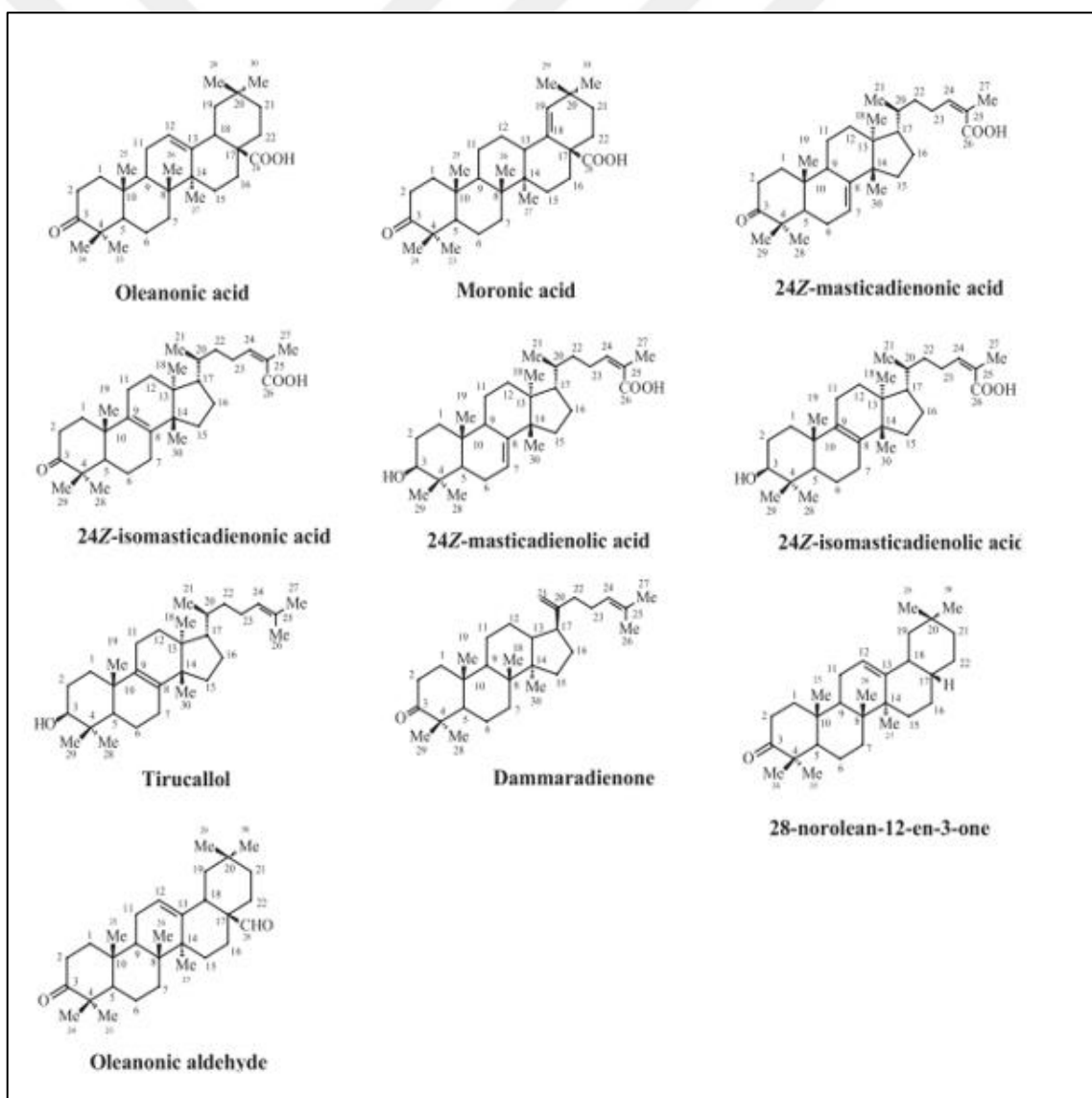


Figure 2.8. Chemical structures of some Triterpenes found in Mastic resin [174]

2.2.2.2. *Synthetic Polymeric Scaffolds*

Synthetic materials have been widely used in tissue engineering applications. When compared to natural polymers, synthetic polymers have several advantages: chemical and mechanical tailorability, bioresorbability, and good physical-mechanical properties such as elastic modulus and degradability. However, synthetic materials are incapable of integrating with host tissues, might lead permanent inflammatory reactions and have limited bioactivity. In order to overcome these, they can be treated with some functional groups like carboxyl, hydroxyl or amino or peptide sequences such as integrin and heparin [9,175]. Poly(α -hydroxy esters), poly(ethylene glycol), poly(NiPAAm), poly(propylene fumarate) and poly(urethane) are the most common synthetic materials used for cartilage tissue engineering.

Poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and their copolymer poly (lactic-co-glycolic acid) (PLGA) are widely used poly(α -hydroxy esters) due to the FDA approval for clinical use and well defined biodegradable characteristics. PLA and PGA can be degraded through their monomers with the hydrolysis of the ester bonds found in the backbones. The degradation products are not cytotoxic and they can be excreted by natural metabolic pathways. PGA is slightly soluble in most of the organic solvents; however its solubility can be increased with the addition of fluorine. When methylated, PLA is more hydrophobic than PGA, but it is soluble in organic solvents [9,56,175,176]. PGA and PLA can be copolymerized with different ratios to form PLGA. For example, higher ratio of lactide (LA) to glycolide (GA) in the copolymer composition leads to an increase in the degradation time, mechanical strength, and hydrophobicity [177]. PLGA has been used for redifferentiation of the dedifferentiated chondrocytes [178], and cartilage tissue reconstruction in both *in vivo* and *in vitro* [179,180].

Poly(ethylene glycol) (PEG) is generally used in the form of hydrogel for cartilage tissue engineering applications. PEG hydrogels can mimic the native cartilaginous tissues by maintaining chondrogenic phenotype [181, 182], and stimulating chondrogenic differentiation of MSCs [183, 184]. They can also be combined with different materials or modified to support cartilage reconstruction [185-187].

Poly(NiPAAm) is a thermo-responsive material which displays a soluble hydrophilic state below 32 °C (its lower critical solution temperature), and an insoluble hydrophobic state above this temperature. It can be used as an injectable *in situ* forming biomaterial in many biomedical implementations [188], including cartilage regeneration. Cho *et al.* constructed a poly(NiPAAm) scaffold that stimulated chondrogenic differentiation of the human MSCs both *in vitro* and *in vivo* [189].

Poly(propylene fumarate) (PPF) is an unsaturated linear polyester which has biocompatible degradation products. Polyurethanes (PURs) contain polymers that demonstrate linear, branched and cross-linked architectures. While PPF has weak mechanical strength, PURs are tough and stable polymers. Both polymers are good candidates for cartilage tissue engineering applications [76,190].

2.2.3. Stimulating Factors

Stimulating factors are the third constituent of the tissue engineering approach, which have been used for inducing, accelerating or enhancing cartilage tissue formation. They can be introduced to the cells through the culture media by microspheres or gene delivery or incorporated into scaffolding materials to promote chondrogenesis. The differentiation of MSCs begins with their interaction with the molecular signals produced by adjacent tissue cells that are transmitted through extracellular or intracellular pathways. The possible ways of triggering differentiation of MSCs involve stem cell surface receptor stimulation through growth factors, cytokines, peptides, and ECM proteins, or direct interaction of cell surface proteins of the adjacent cells. For this reason, the *in vitro* MSC chondrogenic differentiation approaches are based on the knowledge of processes during native cartilage development [34,51].

Transforming growth factor beta (TGF- β), fibroblast growth factor (FGF), bone morphogenic protein (BMP) and insulin-like growth factor (IGF) are widely used stimulating factors for *in vitro* or *in vivo* chondrogenesis. Synergistic and independent effects of these growth factors on cartilage regeneration have been investigated by many researchers.

TGF- β superfamily members are the key regulators of cartilage development. They have essential roles in cartilage formation and maintenance [191]. These growth factors transduce signals through intracellular molecules, SMAD-2 and/or -3, and induce the expression of cartilage specific genes [192]. TGF- β 1, 2 and 3 are the three isoforms of TGF- β that have various effects on different cell types. For instance, while TGF- β 1 controls the cell to cell interactions of condensing progenitor cells [193], TGF- β 2 regulates hypertrophic differentiation, and TGF- β 3 has more stimulative effects on chondrogenic differentiation of MSCs [34]. Moreover, Barry *et al.* showed that TGF- β 2 and TGF- β 3 have more chondroinductive influence over BM-MSCs than TGF- β 1 [194].

BMP also belongs to TGF- β superfamily. The BMP family constitutes 20 polypeptides, which have significant roles in both chondrogenesis and osteogenesis. Several BMPs, especially BMP-2, -4, -6, -7 and -14 are involved in chondrogenesis and cartilage repair. Cartilage formation can be regulated by BMPs either Smad-dependent or Smad-independent pathways. Smad-dependent pathway is initiated upon activation of the Serine/Threonine cell surface receptors, which in turn transfer signals to the Smad1, Smad5, and Smad8. Subsequently, Smad-1, -5 and -8 together form a complex with Smad-4, then the complex translocates to the nucleus where it binds the promoters of target genes to stimulate SOX9, Col2a1, and Runx2 expression. SOX9 is the main regulator of chondrogenesis, therefore, BMP signaling is essential for cartilage formation [28,35,195-197].

Among 22 proteins that appertain to FGF family, FGF-2 is the most studied molecule for cartilage regeneration. FGF-2 exists in the pericellular matrix of the natural cartilage and is used for preserving the chondrogenic phenotype and increasing the cell proliferation thereby resulting in higher ECM production and expedited cartilage repair [28,198].

IGF-1 has various effects on cartilage tissue. It is an indispensable molecule for cartilage homeostasis and has a role in chondrocyte survival and proliferation. Besides, IGF-1 can also stimulate the chondrogenic differentiation, and the synthesis of proteoglycans and type II collagen [28,199].

As potential stimulating factors, small molecules have several advantages over growth factors in terms of stem cell differentiation. First, they can be produced at desired amounts through the synthetic chemistry and various small molecules can be obtained by minor modifications to their chemical structures. Thus, they are cost-effective and tailorable compounds compared to growth factors. Secondly, small molecules are able to act on stem cell differentiation process in rapid and well-controlled manner. The undesired effects of small molecules can be eliminated by specific agonists or antagonists. Lastly, small molecules can affect the behaviors of different types of cells, which lead the use of small molecules in broad-range applications for cell-based tissue engineering [200].

The KGN is a small molecule that promotes chondrogenic differentiation of MSCs (Figure 2.9). It was first discovered by Johnson *et al.* among 22,000 structurally diverse, heterocyclic, drug-like molecules in 2012. In this study, human bone marrow MSC pellet culture was subjected to KGN for 21 days. Upon KGN treatment, cartilage specific proteins including type II collagen, aggrecan, lubricin and SOX9 were highly expressed. Furthermore, they showed the chondroprotective effects of KGN *in vitro* and *in vivo* osteoarthritis (OA) animal model. The molecular mechanism of the chondrogenic induction by KGN was also determined. KGN stimulates chondrogenesis by initiating the nuclear translocation of core-binding factor β subunit (CBF β). CBF β forms a heterodimeric transcription complex along with the runt-related transcription factor (RUNX) family members: RUNX1, -2 and -3. In the cytoplasm, CBF β presents in the resting state by binding its cytoplasmic partner filamin A (FLNA). KGN disrupts CBF β -FLNA complex by binding FLNA, thus releasing CBF β . The liberated CBF β then translocates to nucleus and associates with RUNX1, which in turn stimulates chondrogenic differentiation [201].

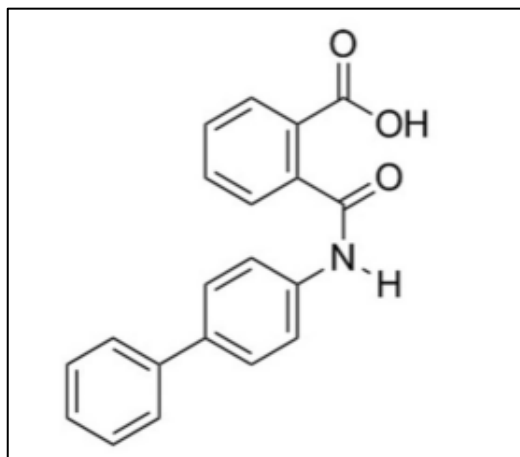


Figure 2.9. Chemical structure of KGN [201]

There have been several studies conducted following the discovery of KGN. Kang and colleagues treated osteoarthritic rats intra-articularly with KGN conjugated chitosan nanoparticles and they found that KGN treated rats showed much less degenerative changes compared to untreated ones [202]. Another group used KGN loaded photo-crosslinkable scaffold for hyaline cartilage regeneration of New Zealand White rabbits. They also showed the capability of KGN to induce human synovium derived mesenchymal stem cells into chondrocytes *in vitro* [203]. Unlikely, Wang *et al.* discovered a different potential of KGN. They demonstrated the induction of collagen synthesis of human dermal fibroblasts by KGN, through the activation of smad4/smad5 pathway [204].

2.3. AIM OF THE STUDY

The aim of this study was to develop cryogels by combining LBG, XG, and MG natural materials with varying concentrations in order to support and promote chondrogenic differentiation of RBM-MSCs. For this purpose, cryogels were chemically and mechanically characterized, release profile of the chondrogenic stimulator KGN from the prepared cryogels were determined, and *in vitro* and *in vivo* evaluations of RBM-MSCs seeded cryogels for chondrogenic differentiation were performed.

3. MATERIALS

3.1. CHEMICALS AND REAGENTS

- Locust Bean Gum from *Ceratonia Siliqua* Seeds (G0753) (Sigma-Aldrich, Germany)
- Xanthan Gum from *Xanthomonas campestris* (G1253) (Sigma-Aldrich, Germany)
- Mastic Gum (G0878) (Sigma-Aldrich, Germany)
- Kartogenin (SML0370) (Sigma-Life Science, Germany)
- Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4500 mg/L) Medium (Gibco-Thermo Fisher Scientific, USA)
- MEM α , GlutaMAX™ Supplement, no nucleosides (Gibco-Thermo Fisher Scientific, USA)
- Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific, USA)
- Sodium azide (Sigma-Aldrich, Germany)
- Sodium Phosphate Monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) (Bioshop, Canada)
- Disodium hydrogen phosphate, anhydrous (Na_2HPO_4) (Sigma-Aldrich, Germany)
- Formaldehyde solution 37% by weight (Riedel-de Haën, Germany)
- Isopropanol (Sigma Aldrich, Germany)
- Potassium bromide (KBr) (Sigma Aldrich, Germany)
- Fetal Bovine Serum (FBS) (Gibco-Thermo Fisher Scientific, USA)
- Penicillin-Streptomycin (10,000 U/mL) (Gibco- Thermo Scientific, USA)
- Dexamethasone (Sigma-Aldrich, Germany)
- L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Santa Cruz Biotechnology, USA)
- Sodium Pyruvate Solution (Sigma Aldrich, Germany)
- 3-Isobutyl-1-methylxanthine (IBMX) (Chem Cruz- Santa Cruz Biotechnology, USA)
- L-Proline (Sigma-Aldrich, Germany)

- Human Transforming Growth Factor β -1 (TGF β -1) Recombinant Protein (Gibco-Thermo Fisher Scientific, USA)
- Corning™ ITS Premix Universal Culture Supplement (Fisher Scientific, USA)
- Indomethacin (Chem Cruz- Santa Cruz Biotechnology, USA)
- Insulin solution human (Sigma-Aldrich, Germany)
- β -Glycerophosphate disodium salt hydrate (Sigma-Aldrich, Germany)
- Alizarin-Red Solution (Merck-Millipore, Germany)
- Rompun (Bayer, Germany)
- Ketalar (Ketamin) (Pfizer, USA)
- Tween® 20 (AppliChem, USA)
- Oil Red O (Sigma-Aldrich, USA)
- ProLong™ Gold Antifade Mountant (Life Technologies-Thermo Scientific, USA)
- NucleoZOL (Macherey-Nagel, USA)
- Binding Buffer MX (Macherey-Nagel, USA)
- Dimethyl Sulfoxide (DMSO), Sterile (Bioshop, Canada)
- Trypsin-EDTA solution (Sigma-Aldrich, Germany)
- Primers for COL2A1, ACAN, SOX9, RUNX1, COL1A1 (Macrogen, South Korea)
- Entellan (Merck, Germany)
- Paraffin Wax (Patolab, Turkey)
- Xylol (Tekkim, Turkey)
- Absolute ethanol (Tekkim, Turkey)
- Distilled water

3.2. KITS

- Alcian Blue PAS Stain Kit (Atom Scientific, UK)
- iScript cDNA synthesis kit (Bio-Rad, USA)
- Maxima SYBR Green, ROX qPCR Master Mix, 2X (Thermo Fisher Scientific, USA)
- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA)

- NucleoSpin RNA, Mini kit for RNA purification (Macherey-Nagel, USA)
- LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Invitrogen-Thermo Fisher Scientific, USA)

3.3. ANTIBODIES

- PE Mouse Anti-Rat CD90 (BD Biosciences, USA)
- PE Hamster Anti-Mouse CD29 (BD Biosciences, USA)
- CD34 Antibody (ICO-115) [Alexa Fluor® 488] (Novus Biologicals, USA)
- FITC Mouse Anti-Rat CD45 (BD Biosciences, USA)
- Collagen II Antibody (5B2.5) [Alexa Fluor® 488] (Novus Biologicals, USA)
- Aggrecan Conjugated Antibody (Alexa Fluor® 647) (Signalway Antibody, USA)

3.4. LABORATORY EQUIPMENTS

- Falcon® Conical Centrifuge Tubes (15 mL) (Corning, USA)
- Falcon® Conical Centrifuge Tubes (50 mL) (Corning, USA)
- Microcentrifuge tubes, 1.5 ml (Isolab, Germany)
- Tissue culture flasks | T75 flask (TPP®, Switzerland)
- Tissue culture flasks | T150 flask (TPP®, Switzerland)
- 12 well cell culture plate (TPP®, Switzerland)
- 24 well cell culture plate (TPP®, Switzerland)
- Microscope cover glasses (Marienfeld, Germany)
- Positively charged microscope slides (Epiglass-Epifarma, Turkey)
- 3 well chamber glass slide (Ibidi, Germany)
- Surgical suture (Ruschmed, Turkey)
- 10 mL Serological Pipette (Jet Biofil, China)
- 25 mL Serological Pipette (Jet Biofil, China)
- Surgical forceps

- Surgical clamps
- Surgical blades
- Sterile syringes
- Forceps

3.5. INSTRUMENTS

- Fluorescence Microscope (Axio Vert.A1, Carl Zeiss, Germany)
- Ultracentrifuge (Avanti® J-25, Beckman Coulter, USA)
- Carbondioxide incubator (Steri-Cycle CO2 Incubator, Thermo Fisher Scientific, USA)
- Laminar flow hood (TELSTAR Bio-II-A, Class II Cabinet, Telstar Life Sciences, Spain)
- Nanodrop (Nanodrop 2000, Thermo Fisher Scientific, USA)
- CFX Touch™ Real Time PCR Detection System (Bio-Rad, USA)
- FACSCalibur™ Flow Cytometry System (BD Biosciences, USA)
- Elisa Plate Reader (Elx800, Bio-Tek, USA)
- Lyophilizator (Savant Modulyo Freeze Drying Systems, Thermo Fisher Scientific, USA)
- Scanning Electron Microscope (EVO, Carl Zeiss, Germany)
- Confocal microscope (LSM 700, Carl Zeiss, Germany)
- Manual Hydraulic Press (Specac, UK)
- ATI Mattson Genesis Series FT-IR Spectrometer (Mattson-Garvin, USA)
- Differential Scanning Calorimetry 131 (Setaram, France)
- DMA Q800 (TA Instruments, USA)
- Instron Universal Testing System 5966 (Instron, USA)
- VNMRs 600 MHz NMR spectrometer (Agilent, USA)
- Microtome (Leica RM2125RTS, Germany)
- Microscope Camera (Leica DFC450, Germany)
- Light microscope (Leica DM2500, Germany)
- Shaking Water Bath (Mettmert, Germany)
- Glass Carbondioxide chamber

4. METHODS

4.1. PREPARATION OF CRYOGELS

LBG (Sigma G0753), XG (Sigma G1253), MG, LBG-XG and LBG-XG-MG cryogels were prepared separately.

LBG was dissolved in distilled water (dH₂O) at different concentrations (1 -5 percent w/v) with continuous stirring between 90-100 °C for 30 minutes. The solution was poured into glass petri-dish and immediately put at -20 °C for 24 hours. Finally, the gels were lyophilized.

XG powder was dissolved in dH₂O with different concentrations ranging between 0.2 percent and 4 percent (w/v) at temperatures between 90-100 °C. Following 30 minutes of incubation, the resulted gels were put directly into freezing compartment (- 20 °C), and incubated there for 24 hours. The cryogels were lyophilized at the final stage.

MG pellets were pounded with mortar and pestle to obtain fine-sized particles. Then, the MG was dispersed in dH₂O with continuous stirring for 30 minutes by maintaining the temperature between 90-100 °C on magnetic stirrer. Afterwards, the solutions were poured into glass petri dishes and stored in the refrigerator at -20 °C for 24 hours. Following this step, the scaffolds were completely dried by lyophilization.

LBG and XG were mixed at the ratio of 1:1 and 2:1 (w/w) with concentrations of 2-3 percent (w/v) in dH₂O to prepare LBG-XG cryogels. After blending, the mixtures were heated to 90-100 °C and stirred for 30 minutes. LBG-XG-MG cryogels were prepared in the same method with LBG-XG cryogels. Briefly, 5 mg/mL and 10 mg/mL MG were mixed with LBG and XG. After dissolving materials in dH₂O, the gels were poured into glass petri-dishes and incubated at -20 °C for 24 hours. The obtained cryogels were dried by lyophilizator and kept at room temperature (RT) for further experiments.

4.2. CHARACTERIZATIONS OF CRYOGELS

4.2.1. Degradation Analysis

The degradation analyses were performed to the cryogels given in Table 4.1. *In vitro* degradation analyses of the scaffolds were performed in centrifuge tubes that had PBS solution containing 0.09 percent sodium azide (pH: 7,5) and incubated at 37 °C in shaking water bath. At the beginning, the scaffolds were weighed, and initial weights of the samples were recorded. The scaffolds were taken out from the water bath at days 7, 15, 30 and 60 for degradation and pH analysis. The pH values of the scaffold-containing PBS solutions were measured to designate pH drop during degradation process. The final weights of the scaffolds were determined after freezing (at -80 °C) and lyophilization steps. The percent weight loss values were calculated by subtracting final weights from the initial weights of the scaffolds.

Table 4.1. The samples selected for degradation analysis

Sample Number	Sample Content
1	%2 (w/v) - 1:1 (w/w) LBG-XG
2	%2 (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG
3	%2 (w/v) - 1:1 (w/w) LBG-XG + 10 mg/mL MG
4	%2 (w/v) - 2:1 (w/w) LBG-XG
5	%2 (w/v) - 2:1 (w/w) LBG-XG + 5 mg/mL MG
6	%2 (w/v) - 2:1 (w/w) LBG-XG + 10 mg/mL MG
7	%3 (w/v) - 1:1 (w/w) LBG-XG
8	%3 (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG
9	%3 (w/v) - 1:1 (w/w) LBG-XG + 10 mg/mL MG
10	%3 (w/v) - 2:1 (w/w) LBG-XG
11	%3 (w/v) - 2:1 (w/w) LBG-XG + 5 mg/mL MG
12	%3 (w/v) - 2:1 (w/w) LBG-XG + 10 mg/mL MG
13	%2 (w/v) LBG
14	%3 (w/v) LBG
15	%2 (w/v) XG
16	%3 (w/v) XG

4.2.2. Scanning Electron Microscopy (SEM) Analysis

The topography of the cryogels was examined by using SEM (Carl Zeiss EVO, Germany) instrument after gold nanoparticle coating via Sputter Coater (Bal-tec SCD 005, Germany). SEM micrographs were used to determine pore size of the scaffolds. Pore diameters of the scaffolds were measured by software which was developed in .NET platform using C# programming language. In the program, the size of 50 randomly selected pores was measured for each sample, and percent size distribution of cryogels was calculated.

4.2.3. Swelling Ratio Analysis

Swelling ratio analyses of cryogels were performed in different swelling solvents (cell culture medium (α -MEM) and PBS pH:7.4) by using following procedure. First, the cryogels were dried and lyophilized until they reached a constant weight. Afterwards, dried cryogels were weighed and incubated into the swelling solvents at 37 °C for 3 hours. At the end of incubation time, the excess surface solution was gently wiped with filter paper and swollen weight values of the scaffolds were recorded. Swelling ratio was calculated using following equation:

$$\text{Percent swelling ratio} = \frac{W_s - W_d}{W_d} \times 100 \quad (4.1)$$

where, W_s is the swollen hydrogel weight and W_d is the dry hydrogel weight at time t .

4.2.4. Fourier-Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR analysis was performed to examine chemical structures of the LBG, XG, LBG-XG, and LBG-XG-MG cryogels. The sample pellets were prepared by mixing KBr with cryogels under approximately 10,000 psi pressure using a manual hydraulic press (Specac, UK). The spectra were recorded from 4,000–400 cm^{-1} region by using ATI Mattson Genesis Series FT-IR spectrometer.

4.2.5. Differential Scanning Calorimetry (DSC) Analysis

DSC analyses were performed to examine thermal properties of LBG, XG and MG containing scaffolds in Table 4.1. The thermograms of the samples were recorded by using SETARAM DSC 131 instrument. Each sample was weighed (8 mg) and put into 120 μ l aluminum pans, and all the measurements were performed between 30-600 $^{\circ}$ C at a heating ramp rate 10 $^{\circ}$ C/minute in the nitrogen atmosphere.

4.2.6. Mechanical Characterization

Tensile, shear and compressive modulus of the cryogels were measured both in dry and wet conditions at RT with dynamic mechanical analyzers (DMA Q800 TA, Instron 5966). Samples were incubated in PBS (1X) for 10 minutes in order to determine tensile, compression and shear modulus values in wet condition. The scaffolds were cut into rectangular shape ($6 - 2 \pm 0.4$ mm) ($n = 3$) to measure tensile properties of the samples. The strain rate of the instrument was arranged to 5 mm/minute for tensile analysis. Shear modulus values were determined under the strain rate 0.08 N/minute using shear sandwich clamp. The dimensions of the samples were 2.1 ± 0.5 mm in thickness, $9 \text{ mm} \pm 0.5$ mm in length and width. The compression analysis was performed under strain 2 mm/minute to rectangular prism samples with dimensions 7 ± 0.5 mm in thickness, 9 ± 0.5 mm in width and 10 ± 0.5 mm in length.

4.2.7. Release Studies of KGN

Release studies of KGN from the cryogels was used for chondrogenic differentiation of rat bone marrow mesenchymal stem cells (RBM-MSCs). KGN small molecule was entrapped in LBG, XG, LBG-XG and LBG-XG-MG cryogels. Briefly, a stock solution (12.5 mg/mL) was prepared by dissolving KGN in dimethyl sulfoxide (DMSO). Then, KGN stock solution was diluted to 150 μ M with dH₂O and loaded into each dry scaffold. Afterwards, KGN loaded scaffolds were put into the release medium (1 mL PBS) separately. Wavelength scan analysis was performed to detect optimal wavelength for KGN, and 278 nm was chosen for the subsequent release

studies. In order to measure absorbance values at 278 nm, samples were taken from the release media of each scaffold at days 1, 3, 7, 14 and 21. The amount of released KGN was calculated according to the equation obtained from the calibration curve of KGN.

4.2.8. Proton Nuclear Magnetic Resonance (^1H -NMR) Spectroscopy

The ^1H -NMR analysis was performed to the samples enumerated as 4, 5, 7, 8, 10, and 11 in Table 4.1, and LBG, XG and MG, separately. The ^1H -NMR spectra of the samples were determined by using the Agilent VNMRS 600 MHz NMR spectrometer by tuning at 9615.4 Hz, after the samples were dissolved in deuterated water (D_2O).

4.3. *IN VITRO* CELL-CRYOGEL ANALYSES

4.3.1. Isolation and Characterization of RBM-MSCs

MSCs were isolated from the bone marrow of 6-8 weeks old male Sprague-Dawley rats. The study was approved by the Yeditepe University Experimental Animals Ethical Committee. The femoral and tibial bones were separated from the rats and transferred in a medium (MEM- α (1X) + GlutaMAXTM, Gibco, ABD) containing 10 percent Penicillin-Streptomycin (10,000 U/mL). The bones were cut from the metaphyseal parts and the bone marrow was ejected with the help of a sterile syringe under laminar flow cabinet (Telstar, Bio-II-A, Spain). The extracted bone marrow was centrifuged at 2,000 rpm for 5 minutes. The resulting pellet was dissolved in the growth medium (MEM- α (1X)+GlutaMAXTM with 10 percent Fetal Bovine Serum (FBS), 100 unit/mL penicillin and 100 unit/mL streptomycin), and incubated in an incubator at 37 °C, in 5 percent CO_2 , and 90 percent humidity. The culture medium of the RBM-MSCs was replenished every other day.

When the cells reached to passage 3, the immunophenotypic characterization of the cells was performed through flow cytometry analysis. In this context, RBM-MSCs were trypsinized and counted with cell counter. The cells were dissolved in PBS and distributed to flow cytometry

tubes (500,000 cells/tube). Afterwards, the cells were labeled with specific cell surface markers CD90 (BD Pharmingen, USA), CD 29 (BD Biosciences, USA), CD 34 (Novus Biological, USA), and CD45 (BD Biosciences, USA) for 1h at RT. After centrifugation step, the cells were resuspended in 400 μ L PBS and analyzed via flow cytometer (FACSCalibur, Becton Dickinson, USA) to verify their stem cell nature.

Furthermore, osteogenic, chondrogenic, and adipogenic capability of the cells were confirmed by Alizarin red, Alcian Blue and Oil Red O stainings, respectively. Characterization studies of RBM-MSC differentiation were performed at days 7, 14 and 21. At first, the cells were seeded into each well of 12-well plates (25,000 cells/well) and they were incubated in osteogenic, chondrogenic and adipogenic medium. For osteogenic differentiation medium, MEM- α (1X) + GlutaMAX™ containing 10 percent FBS, 1 percent penicillin/streptomycin, 0.1 μ M dexamethasone, 10 mM β -glycerophosphate disodium salt hydrate, and 50 μ M L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate was used. Chondrogenic differentiation medium was prepared in high glucose DMEM (4,5 g/L) containing 1 percent penicillin/streptomycin, 0.1 μ M dexamethasone, 1% v/v ITS premix, 50 μ g/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 40 μ g/mL proline, 10 μ g/mL sodium pyruvate, 10 ng/mL TGF- β . In order to prepare adipogenic differentiation medium, 0.1 μ M dexamethasone, 0.5 mM IBMX, 100 μ M indomethacin and 10 μ g/mL insulin were mixed into high glucose DMEM (4.5 g/L) containing 1% penicillin/streptomycin and 10 percent FBS. Control groups were incubated in growth medium containing the basal medium with 10 percent FBS and 1 percent penicillin/streptomycin.

At 7th, 14th and 21st days of osteogenic induction, Alizarin red staining was carried out in order to determine mineralization. Alizarin red stains calcium produced by differentiated RBMSCs. At first cells were fixed with 3.7 percent formaldehyde solution (v/v) for 1 hour at RT. Then, Alizarin red stain was added into the each well containing cells and incubated for 45 minutes at RT. After the washing steps, each well was investigated under light microscope and the photographs of the wells were taken to confirm osteogenic differentiation of RBMSCs.

Alcian Blue staining was performed at days 7, 14 and 21 after chondrogenic induction. Initially, the cells were fixed with 3.7 percent formaldehyde for 1 hour. Alcian blue stain was dropped into each well and waited for 10 minutes. After washing steps, the cells were treated with 1 percent periodic acid for 5 minutes and then Schiff reagent for 20 minutes. Then, the cells were washed under running water and stained with Haemalum Mayer for 1 minute. Finally, the cells were observed microscopically, and the photographs of each well were taken under different magnifications.

Oil Red O staining technique was performed to visualize oil droplets containing triglycerides, neutral lipids and cholesterol esters, produced by adipocytes. Initially, the cells were washed with PBS and then fixed with 3.7 percent formaldehyde for 1 hour. Afterwards, 60 percent isopropanol solution was added onto each well and waited for 5 minutes. The cells were treated with Oil Red O working solution for 5 minutes at RT and finally they were rinsed with dH₂O. Each well was investigated under the light microscope and the photographs of the wells were taken to indicate adipogenic differentiation of rat bone marrow cells (RBMCs).

4.3.2. Effect of KGN on Chondrogenic Differentiation

The effect of KGN on cells isolated from rat bone marrow (RBM) was investigated via Alcian Blue staining. The cells were seeded on 12 well plates at a density of 25,000 cells/well. Cells were incubated in KGN containing growth medium, chondrogenic medium (CM) (DMEM (4,5 g/L), 1 percent penicillin/streptomycin, 0.1 μ M dexamethasone, 1% v/v ITS premix, 50 μ g/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 40 μ g/mL proline, 10 μ g/mL sodium pyruvate, 10 ng/mL TGF- β), and KGN containing CM for 21 days. Three different concentrations of KGN (100 nM, 150 nM and 200 nM) were used to determine the effect of KGN on cells. Alcian blue staining was performed at days 7, 14, and 21, then images were taken under the light microscope.

4.3.3. Cell Viability Analysis

4.3.3.1. CellTiter 96® AQueous One Solution Cell Proliferation Assay

Cell viability analysis was performed to determine RBM-MSCs' viability on cryogels. Following cryogel preparation, sterilization process of the bottom and the top surfaces of the scaffolds were carried out under UV-light for 1 hour/each surface. The RBM-MSCs were trypsinized and centrifuged at 2,000 rpm for 5 minutes and 200,000 cells were seeded into each scaffold. The cell-seeded scaffolds were incubated in MEM- α (1X) + GlutaMAX™ medium with 10 percent FBS, 100 unit/mL penicillin and 100 unit/mL streptomycin at 37 °C in 5 percent CO₂ incubator throughout 21 days for MTS cell viability analysis. Medium was refreshed twice a week.

CellTiter 96® AQueous One Solution Cell Proliferation Assay was performed at days 3, 7, 14 and 21. Briefly, cell-laden scaffolds were incubated in a mixture of medium/MTS assay reagent (5:1 (v/v)) for 2 h at 37 °C in 5 percent CO₂ incubator. Afterwards, 200 μ L of the mixture was transferred into 96-well plate and the absorbance values were measured at 490 nm using Elisa Plate Reader.

4.3.3.2. Live/Dead® Cell Viability Assay

Calcein AM (excitation/emission~494/517 nm)– Ethidium Homodimer-1 (excitation/emission~528/617 nm) fluorescent dyes were used to observe live and dead cells inside the cryogels. Passage 3 RBM-MSCs were seeded (400,000 cells/cryogel) into the cryogels and incubated in MEM- α (1X) + GlutaMAX™ medium with 10 percent FBS, 100 unit/mL penicillin and 100 unit/mL streptomycin for 21 days. In order to perform live/dead assay, fluorescent dyes were diluted to 2 μ M with PBS from their stock solutions. Cryogels were incubated in dye solutions for 1h, at RT in dark. Afterwards, cryogels were observed under fluorescence microscope at days 3, 7, 14, and 21.

4.3.4. Chondrogenic Differentiation Capacity of RBM-MSCs in Cryogels

4.3.4.1. Alcian Blue Staining

Alcian blue staining was used to determine cartilage specific ECM molecules. Cell seeded cryogels (400,000 cells/cryogels) were incubated in CM (high glucose DMEM (4.5 g/L) containing 1 percent penicillin/streptomycin, 0.1 μ M dexamethasone, 1% v/v ITS premix, 50 μ g/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 40 μ g/mL proline, 10 μ g/mL sodium pyruvate, 10 ng/mL TGF- β) and in MEM- α (1X)+GlutaMAX™ medium with 10 percent FBS, 100 unit/mL penicillin and 100 unit/mL streptomycin containing 150 nM KGN, respectively. Cell-free cryogels were used as control groups. MSC seeded cryogels that were incubated in two different differentiation mediums, were stained using Alcian Blue–Pas Staining Kit pH:2.5 (Atom Scientific, USA). Stainings were performed according to the manufacturer's protocol.

4.3.4.2. Total RNA Isolation

Chondrogenic differentiation capacity of RBM-MSCs seeded into cryogels was measured via Real Time PCR method. Cells were trypsinized at passage 2 and seeded into cryogels (2×10^6 cells/cryogel). Chondrogenic induction was carried out 3 days later with CM and KGN (150 nM) containing medium. Cell-laden cryogels were also incubated in growth medium (MEM- α (1X)+GlutaMAX™ medium with 10 percent FBS, 100 unit/mL penicillin and 100 unit/mL streptomycin) in order to compare chondrogenic effects of differentiation mediums with the growth medium. NucleoZOL and Machery-Nagel RNA Isolation Kit were combined to isolate RNA on days 3, 7, 18 and 28 from cell-laden cryogels. Briefly, each cryogel was incubated into 1 mL NucleoZOL for 20 minutes, and then centrifuged at 12,000 x g for 15 minutes. After Buffer MX was mixed with the supernatant part of the sample, the mixture was transferred to NucleoSpin® RNA column. Washing steps were performed (Buffer RA3) subsequent to second centrifugation step (8,000 x g for 45 seconds). Finally, RNA was eluted with 60 μ L RNase-free H₂O. The amount of RNA was measured via NanoDrop™ spectrophotometer.

4.3.4.3. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The RNA isolation was followed by cDNA synthesis. RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Reaction mix (5X, 4 µL), reverse transcriptase (1 µL), nuclease free water and RNA template were mixed and incubated at 25°C for 5 minutes, 46°C for 20 minutes, and finally 95°C for 1 minute. Afterwards, cDNAs were used as templates for real time PCR reaction. Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (Thermo Scientific, USA) was used to determine the expressions of SRY-related high-mobility-group box 9 (SOX9), type II collagen (COL2A1), Aggrecan (ACAN), Runt-related transcription factor 1 (RUNX1), type I collagen (COL1A1), and housekeeping gene Beta-actin (ACTB) (Table 4.2). Reaction mixture was prepared for each primer according to Table 4.3 and Cq values were obtained using iCycler, CFX96 Touch Real Time PCR Detection System (BIO-RAD, USA). The data acquisition were performed according to the protocol in Table 4.4. The results were calculated as fold change using $2^{-\Delta\Delta Cq}$ method.

Table 4.2. Primer Sequences used for quantitative Real Time PCR

Primers		Sequence (5'→3')	Annealing Temperature	Product Length
SOX9	F	CTCCTACTACAGCCACGCAG	60 °C	529 bp
	R	AAGTCCAAACAGGCAGGGAG		
COL2A1	F	CACCGCTAACGTCCAGATGAC	59 °C	276 bp
	R	GGAAGGCGTGAGGTCTTCTGT		
ACAN	F	CATTGCGACGGGAGCAGCCA	61 °C	133 bp
	R	TGGGGTCCGTGGGCTCACAA		
RUNX1	F	CCAAACTCTGAAAGCGAGGC	60 °C	300 bp
	R	CTGGCATCTACGGGATACG		
COL1A1	F	GATGGACTCAACGGTCTCCC	60 °C	580 bp
	R	CAGGATCGGAACCTTCGCTT		
ACTB	F	AGGGAAATCGTGCGTGACAT	60 °C	532 bp
	R	CAGCTCAGTAACAGTCCGCC		

Table 4.3. Quantitative Real Time PCR reaction mix

Reagents	Volume
Maxima SYBR Green/ROX qPCR Master Mix (2X)	12.5 µl
Forward Primer (0.3 µM)	0,75 µl (from 10 µM primer stock)
Reverse Primer (0.3 µM)	0,75 µl (from 10 µM primer stock)
Template DNA ≤500 ng	2 µl
Water, nuclease-free	9 µl
Total Volume	25 µl

Table 4.4. Data acquisition protocol

Step	Temperature (°C)	Time	Number of Cycles
Pre-treatment	50	2 minutes	1
Initial Denaturation	95	10 minutes	1
Denaturation	95	15 seconds	55
Annealing	59,60,61	30 seconds	
Extension	72	30 seconds	

4.3.4.4. Immunocytochemistry Analysis

Immunocytochemistry analysis was performed to observe type II collagen and aggrecan molecules synthesized by RBM-MSCs seeded (10^6 cells/cryogel) in cryogels upon KGN treatment on days 7 and 28. First, cell-laden cryogels were incubated in 3.7 percent formaldehyde solution prepared in PBS for 1h, at RT. Then, cryogels were washed with PBS and incubated in 0.1 percent Tween-20 containing PBS solution for 30 minutes. Cryogels were treated with 3 percent FBS dissolved in PBS for 30 minutes in order to avoid non-specific binding of antibodies. After washing cryogels with PBS, Alexa Fluor[®] 488 (excitation:490 nm/emission:525 nm) conjugated type II collagen monoclonal antibody (Novus Biologicals, USA) was dissolved in PBS solution (1:200 dilution) containing 1.5 percent FBS. Then, cryogels were incubated overnight at 4 °C in type II collagen antibody solution. Next day, cryogels were washed with PBS and treated overnight with Alexa Fluor[®] 647 (excitation:650 nm/emission:665 nm) conjugated Aggrecan monoclonal antibody (1:200 dilution) (Signalway Antibody, USA) at 4 °C. Finally, in order to observe nuclei of the cells, 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1:1,000 dilution) was added on cryogels and incubated for 1h at RT in dark. All cryogels were transferred to the well chamber slides, and ProLong[™] Diamond Antifade Mountant (Invitrogen[™], USA) was applied to these samples. Confocal microscope was used to examine type II collagen and aggrecan labeled cells in cryogels.

4.4. IN VIVO ANALYSIS OF CELL-CRYOGEL INTERACTION

According to the results of cryogel characterization and *in vitro* studies, 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels were used for *in vivo* analysis. The classification of the cryogels that was implanted to animals were done as in Table 4.5 during *in vivo* studies. Male 16 weeks old Sprague-Dawley rats were used to investigate ectopic chondrogenesis of RBM-MSCs seeded in cryogels (Figure 4.1 a). For this purpose; dorsal sides of the Sprague-Dawley rats were shaved upon anesthetization with Ketamin (80 mg/kg) and Rompun (6 mg/kg). Afterwards, the incision sites were cleaned using antiseptic solution. A 2 cm of upper midline straight incision was made with a scalpel on dorsal rat skin to implant cryogels subcutaneously (Figure 4.1 b). The wound was closed by applying simple interrupted suture using 3-0 Prolene. Finally, rats were euthanized after 4, and 8 weeks of implantation. Then, cryogels were removed and put into 50 ml centrifuge tubes filled with 10 percent neutral buffered formalin for the fixation of samples for further histological investigation (Figure 4.1 c, d).

Cell-laden cryogels were embedded in paraffin upon 5 days incubation in 10 percent neutral buffered formalin for histological analysis. Then, paraffin blocks were sectioned using microtome (Leica RM2125RTS, Germany) and put on poly-l-lysine microscope slides. Sections were dewaxed in xylene and rehydrated through ethanol solutions with gradually decreasing concentrations. Afterwards, sections were stained with Alcian Blue (pH:2.5) for 20 seconds and mounted with Entellan. Images were taken with a digital camera (Leica DFC450, Germany) attached to a light microscope (Leica DM2500, Germany).

Table 4.5. Animal groups for *in vivo* analysis

Group Number	Groups	Number of animals	
		4 weeks	8 weeks
1	3 % (w/v) 1:1 (w/w) LBG-XG	3	3
2	3 % (w/v) 1:1 (w/w) LBG-XG + RBM-MSCs	3	3
3	Kartogenin loaded 3 % (w/v) 1:1 (w/w) LBG-XG	3	3
4	Kartogenin loaded 3 % (w/v) 1:1 (w/w) LBG-XG + RBM-MSCs	3	3
5	3 % (w/v) 1:1 (w/w) LBG-XG + 5 mg/mL MG	3	3
6	3 % (w/v) 1:1 (w/w) LBG-XG + 5 mg/mL MG + RBM-MSCs	3	3
7	Kartogenin loaded 3 % (w/v) 1:1 (w/w) LBG-XG + 5 mg/mL MG	3	3
8	Kartogenin loaded 3 % (w/v) 1:1 (w/w) LBG-XG + 5 mg/mL MG + RBM-MSCs	3	3

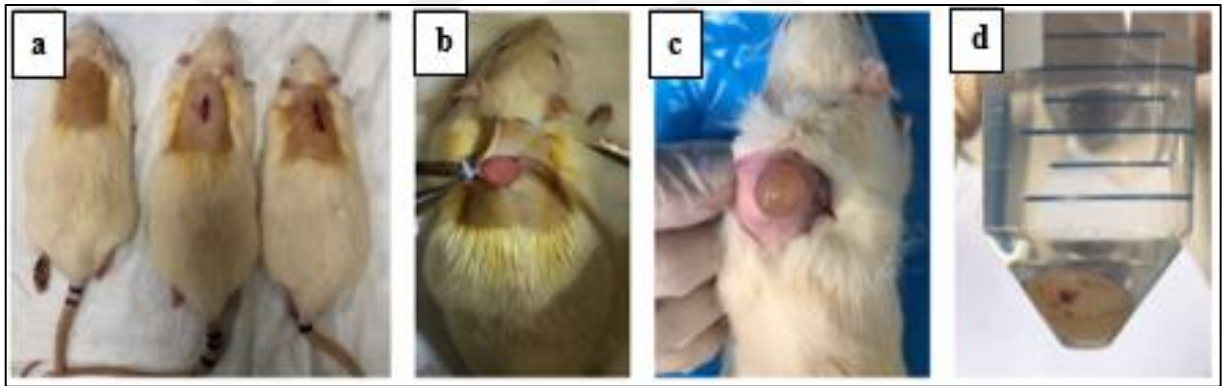


Figure 4.1. (a) Sprague-Dawley rats during operation, (b) implantation of cryogel to the dorsal side of the rats, (c) cryogel taken out from the rat, (d) cryogel inside formalin solution

4.5. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software by one-way or two-way Analysis of variance (ANOVA) followed by Tukey test for multiple comparisons. All data were presented as the mean and standard deviation (SD). The assays were repeated in three independent experiments, and the data having $p < 0.05$ was accepted as statistically significant.

5. RESULTS AND DISCUSSION

5.1. CHARACTERIZATIONS OF CRYOGELS

5.1.1. Degradation Analysis

Degradation of scaffolds is an important phenomenon due to its effect on cells. The ideal scaffold degradation should be proportional to ECM production and deposition [205]. The degradation profile can change diffusion rate of nutrients, interaction of cells with scaffolds, and ECM organization. It was found that degradability results in enhanced neocartilage formation and ECM matrix deposition by MSCs seeded in hydrogels [206].

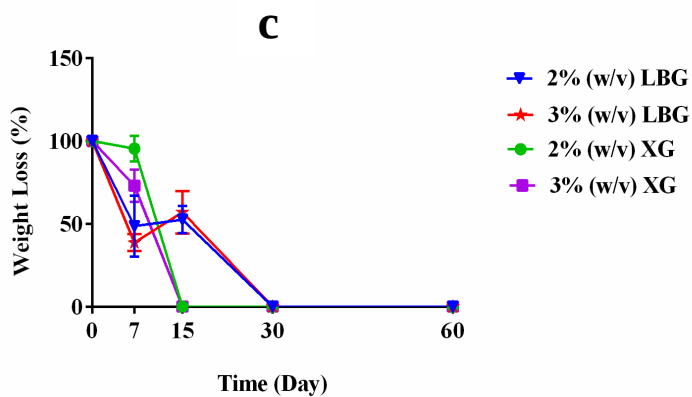
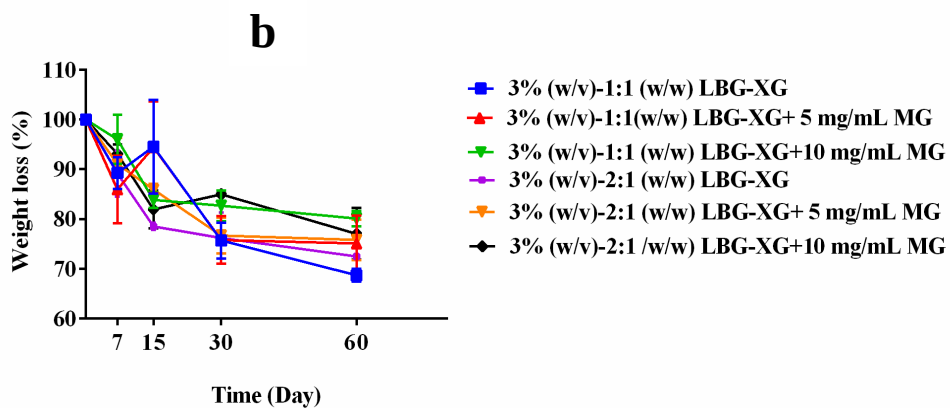
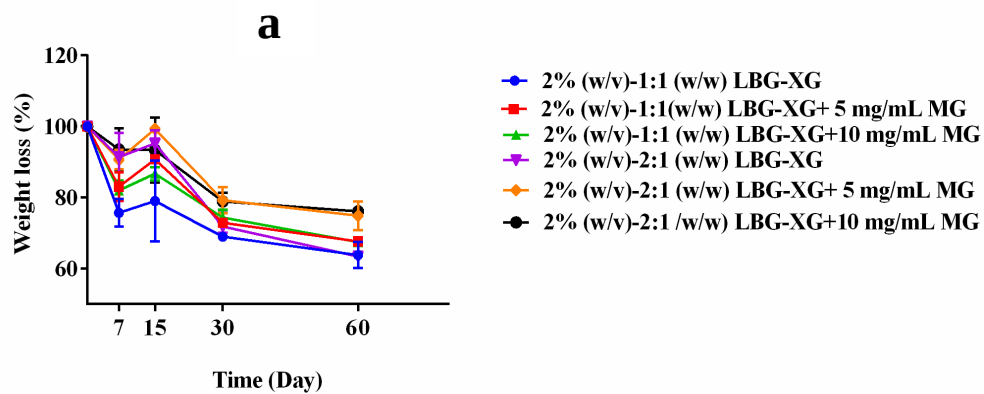
In this study, it was observed that 2 percent (w/v) LBG-XG containing cryogels had higher weight loss (between 24 percent-37 percent) than 3 percent (w/v) LBG-XG containing cryogels (between 23 percent-32 percent) at the end of 60 days (Figure 5.1 a, b, c). While the highest weight loss was observed in the samples of 2 percent (w/v) - 1:1 (w/w) LBG-XG and 2 percent (w/v) - 2:1 (w/w) LBG-XG cryogels (36.23 percent and 36.62 percent respectively), the lowest weight loss was observed in the sample 3 percent (w/v) - 1:1 (w/w) LBG-XG + 10 mg/mL MG (19.92 percent). Regarding these results, it can be concluded that 3 percent samples were more durable than 2 percent samples. The degradation rate of the cryogels were decreased with increasing MG concentration, except the one containing 2 percent (w/v) - 1:1 (w/w) LBG-XG. Concentration increase in MG did not have any effect on degradation rate of 2 percent (w/v) - 1:1 (w/w) LBG-XG cryogels. Complete degradation of LBG and XG cryogels was observed at days 30 and 15, respectively.

The pH values of the solutions containing cryogels were also determined during degradation analysis. Low pH values at the implanted area of the scaffold is an undesirable outcome since the viability of mesenchymal stem cell decreases at acidic environment [207]. For this reason,

the pH changes of the environment due to the degradation products of the scaffolds should be investigated.

When the samples in Table 4.1 compared, the 3 percent (w/v) -1:1 (w/w) LBG-XG+10 mg/mL MG sample had the lowest pH value (7.01) at the end of 60 days (Figure 5.1 d, e, f). The highest pH was found in the sample of 2 percent LBG (w/v) (7.32). The pH values of 2 percent (w/v) LBG-XG samples were slightly higher than the pH values of 3 percent (w/v) LBG-XG samples. The reason of that might be the excess amount of the acidic degradation products of XG (such as glucuronic acid and pyruvic acid) in 3 percent (w/v) LBG-XG samples. When all the pH values were examined, it was determined that none of the pH values dropped under 7.0. The physiological pH values of the human body change between 7.35 and 7.45 [208]. However, it was found that pH values less than 7.0 decreases the MSC viability, and pH values ranging 7.4 to 8.0 increase MSC proliferation and viability [209]. Similarly, Wuertz et al. showed that acidic environment decreased RBM-MSC viability and proliferation, and inhibited aggrecan gene expression [210]. For this reason, it can be concluded that pH values of the environment upon degradation of cryogels were suitable for RBM-MSCs viability and proliferation.

In the light of the information obtained from the degradation results, it was decided to be continued with the blend cryogels since the LBG and XG cryogels were degraded faster compared to the blend ones.



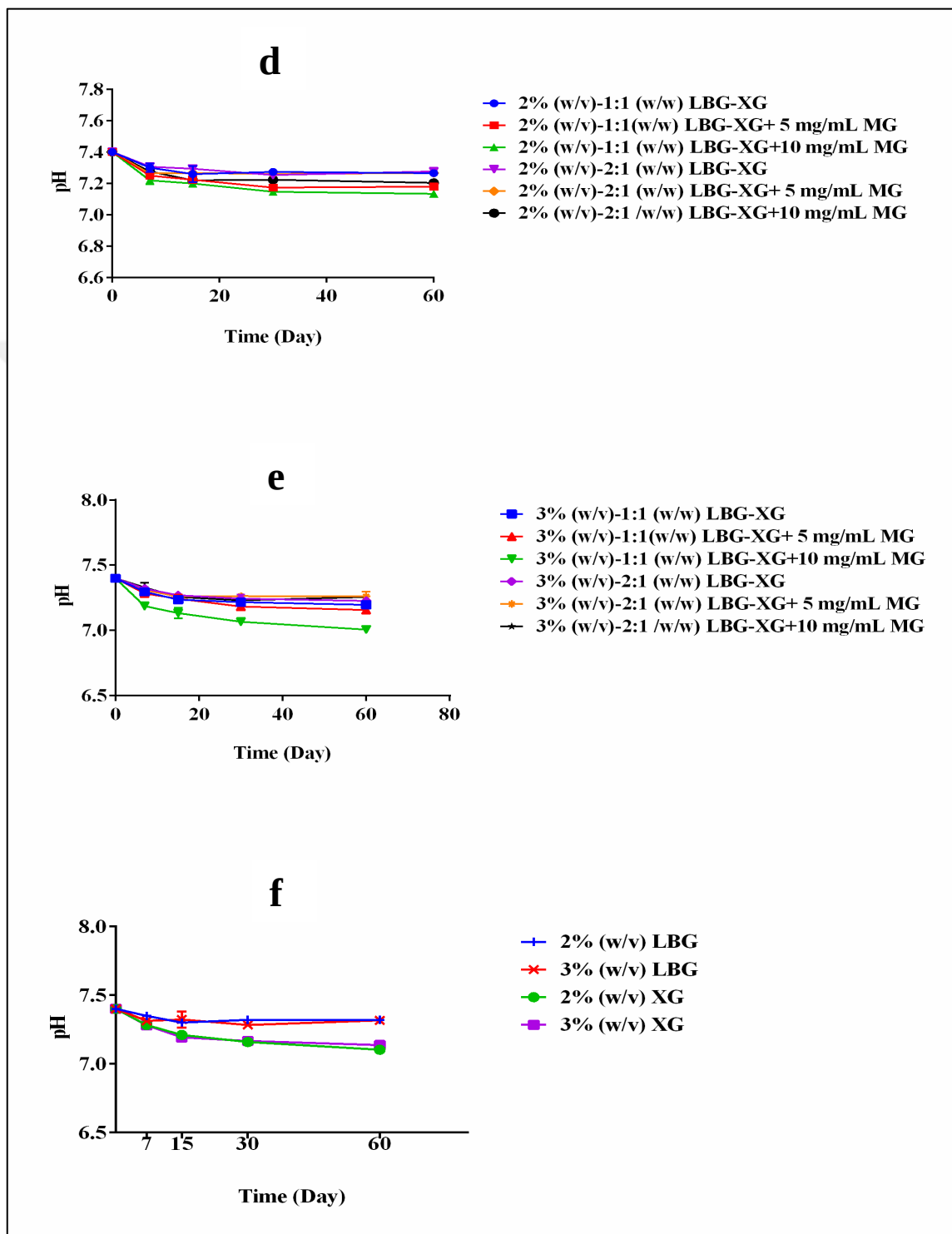


Figure 5.1. Degradation analysis of cryogels. (a,b,c) Percent weight loss of cryogels, (d,e,f) pH values of cryogels during degradation

5.1.2. SEM Analysis

Morphological and architectural structures of cryogels were investigated via SEM. This analysis revealed that LBG-XG, and LBG-XG-MG cryogels had interconnected pore structure (Figure 5.2). The amount of MG and concentration difference in LBG-XG blends did not have any effect on the morphology of cryogels. It is known that interconnectivity and pore size are important for cell attachment, growth, migration and metabolism [211]. In addition, Hwang *et.al.* showed the importance of interconnected pore structure on equal distribution of GAG molecules produced by cells through the whole scaffold [151]. For this reason, the pore sizes of the materials were analyzed using SEM micrographs and their pore size distributions were determined by a pore analysis program written in C-Sharp programming language. The measurements were categorized as the number of pores corresponding to 0-100 μm , 100-200 μm , 200-400 μm and ≥ 400 μm ranges. The results were shown as percent pore size distribution in Figure 5.3.

According to Figure 5.3, all cryogels had pore size under 400 μm except the one with 2 percent (w/v) – 1:1 (w/w) LBG-XG + 10 mg/mL MG. Chondrogenic differentiation, especially proteoglycan production was proven to be increased under the pore size 400 μm [212, 213]. Another observation was that 3 percent (w/v)- 2:1 (w/w) LBG-XG containing samples had pores mostly in the range of 100-200 μm (58-66 percent). Cryogels having 2 percent (w/v) - 1:1 (w/w) LBG-XG, 2 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG, 3 percent (w/v) - 1:1 (w/w) LBG-XG, and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG concentrations had more homogenous percent distribution for pore sizes in the range of 0-100 μm , and 100-200 μm than the rest of the cryogels. When pore size of all cryogels investigated, it was found that 2:1 (w/w) LBG-XG containing samples had larger pores than the one with 1:1 (w/w) LBG-XG. Therefore, it can be concluded that the pore size was increased with increasing LBG concentration. Considering these results, cryogels might be able to provide suitable environment for chondrogenic differentiation of RBM-MSCs.

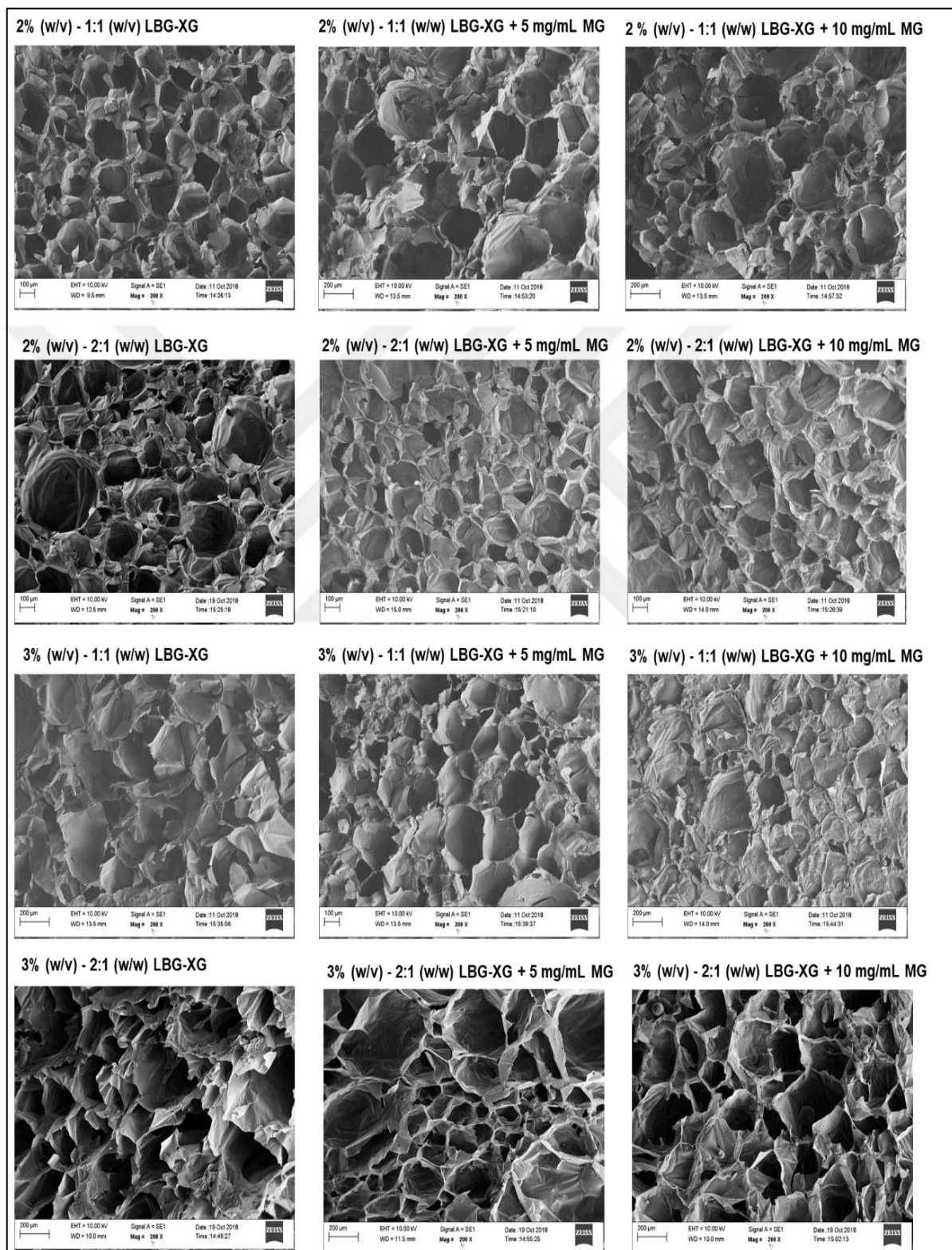


Figure 5.2. SEM micrographs of cryogels (200 X magnification)

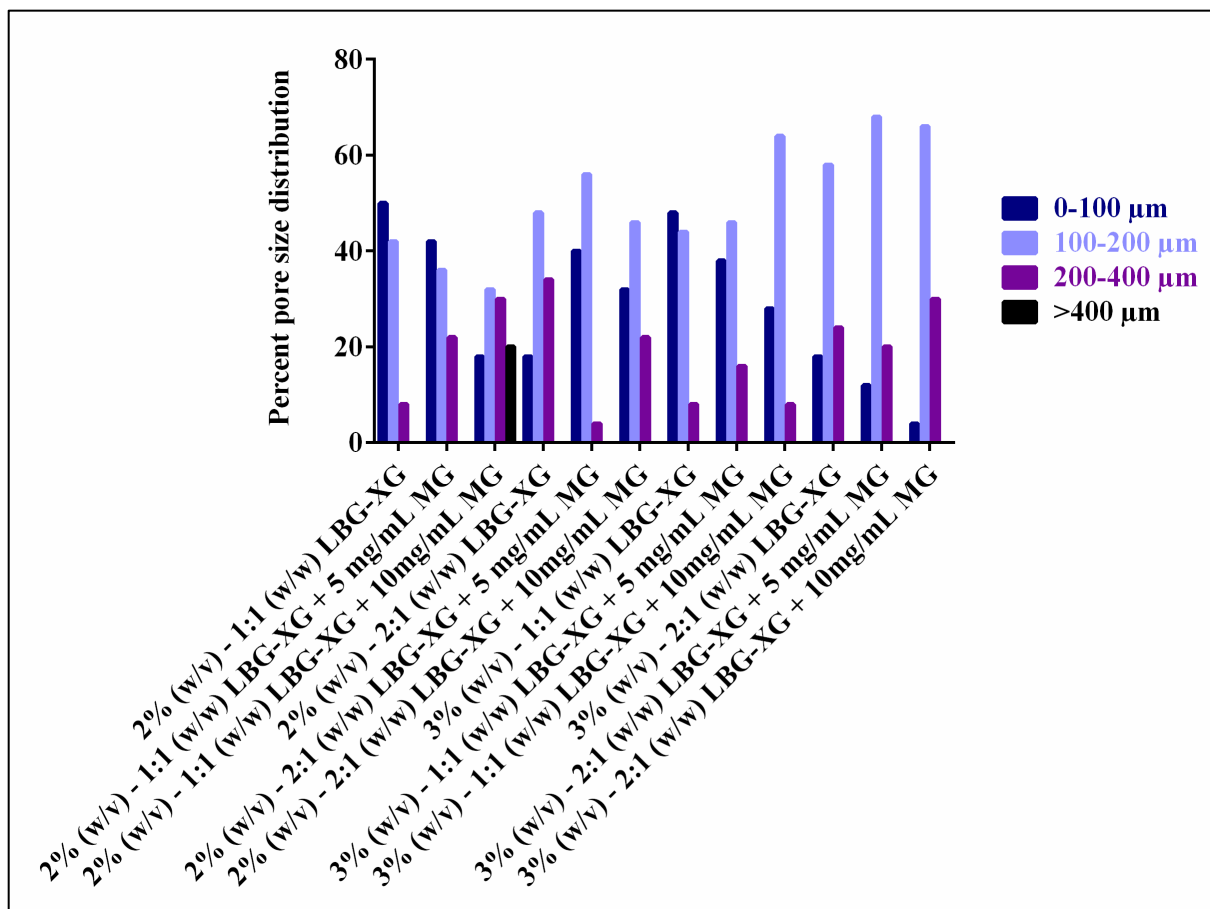


Figure 5.3. Percent pore size distribution of cryogels

5.1.3. Swelling Ratio Analysis

Percent swelling ratio values of cryogels incubated in cell culture media (MEM α , GlutaMAX™) and PBS were determined separately. Swelling values of cryogels incubated in media decreased with increasing MG concentration (Figure 5.4 a). There were nearly 50 percent decrease between percent swelling ratio values of LBG-XG and 10 mg/mL MG containing LBG-XG cryogels. The change in the amount of LBG did not have any effect on the swelling ratio of the cryogels. When percent swelling ratio values of the cryogels incubated in PBS were investigated, no significant differences was observed between MG containing and MG free samples except the one with 2 percent (w/v) – 1:1 (w/w) LBG-XG (Figure 5.4 b). The swelling ratio values significantly decreased with MG addition to the 2 percent (w/v) – 1:1 (w/w) LBG-XG cryogels. Considering the results obtained from two different swelling solvents, it was thought that the interconnected pore structure could be the reason of high swelling rates. This pore structure is important for cell migration, intercellular interaction, and nutrient and substance exchange [211]. It is known that scaffolds with low porosity and small pore size and therefore low swelling ratios have negative affect on cell migration and interaction [214]. In addition to these, it was determined that chondrocyte and mesenchymal stem cell seeded scaffolds with high swelling ratios produced more cartilage specific ECM [214-216].

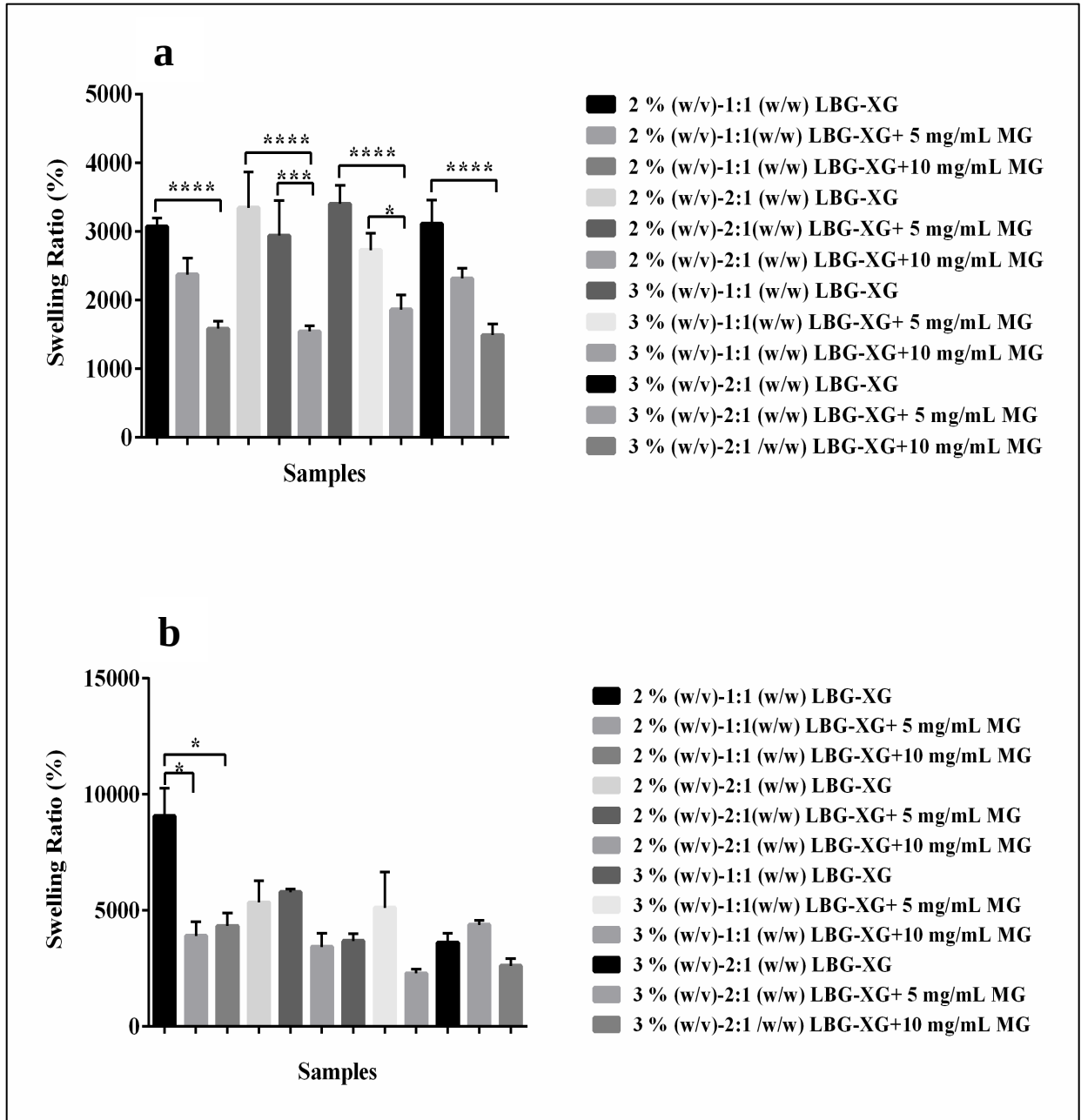


Figure 5.4. Percent swelling ratios of cryogels incubated in (a) MEM α , GlutaMAX™, (b) 1X PBS (* $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

5.1.4. FT-IR Analysis

FT-IR analysis was performed in order to examine the chemical structures of the cryogels. All the FT-IR spectra have a broad peak between $3,200\text{ cm}^{-1}$ – $3,600\text{ cm}^{-1}$ (mostly around $3,400\text{ cm}^{-1}$) which shows O-H stretching [217]. Hydroxyl groups are found in the structures of both LBG and XG, therefore the broad peaks between $3,200\text{ cm}^{-1}$ – $3,600\text{ cm}^{-1}$ were related with the chemical structures of the polymers. The peak around $2,900\text{ cm}^{-1}$ ($2,900\text{ cm}^{-1}$ - $2,940\text{ cm}^{-1}$) indicates –CH stretching vibration and axial deformation of CH_2 [218]. The C-H peak around $2,870\text{ cm}^{-1}$ became more apparent with MG addition in all sample sets [Figures 5.5, 5.6, and 5.7].

Figure 5.5 a, b, c show the FT-IR spectra of LBG, XG, and MG, respectively. The IR bands between $873\text{--}876\text{ cm}^{-1}$ in Figures 5.5 a, 5.6 and 5.7, and the bands between $893\text{--}897\text{ cm}^{-1}$ in Figure 5.5 b were correlated with α and β forms of galactose ($871 \pm 7\text{ cm}^{-1}$) and mannose ($876 \pm 9\text{ cm}^{-1}$) [219]. In polysaccharides, the bands between $1,200\text{ cm}^{-1}$ - 970 cm^{-1} are generally associated with C-C and C-O stretching in pyranoid ring and C-O-C stretching of glycosidic bonds. Apart from the peaks that mentioned above, the peaks around $1,640\text{ cm}^{-1}$ in Figure 5.5 was attributed to the existence of water molecules in LBG, XG and MG. The small peak at $1,150\text{ cm}^{-1}$ indicated C-O-C asymmetrical stretching in the structure of LBG and the peaks around $1,027\text{ cm}^{-1}$ – $1,028\text{ cm}^{-1}$ were the signs of C-O stretching [220]. According to the Figure 5.6 and 5.7, the peaks at $1,731\text{ cm}^{-1}$ and $1,614\text{ cm}^{-1}$ indicated stretching of carbonyl (C=O) groups [221, 222], and the peaks at $1,731\text{ cm}^{-1}$ increased proportionally with MG addition. On the other hand, the peak around $1,032\text{ cm}^{-1}$ in XG indicated the existence of acetal groups in XG (Figure 5.5 b) [223].

The data obtained from the FT-IR showed that all scaffolds had the characteristic peaks of the polysaccharides and had a change in some peaks after blending two polymers. The preservation of characteristic peaks of LBG, XG and MG after mixing all polymers indicated the success of blending.

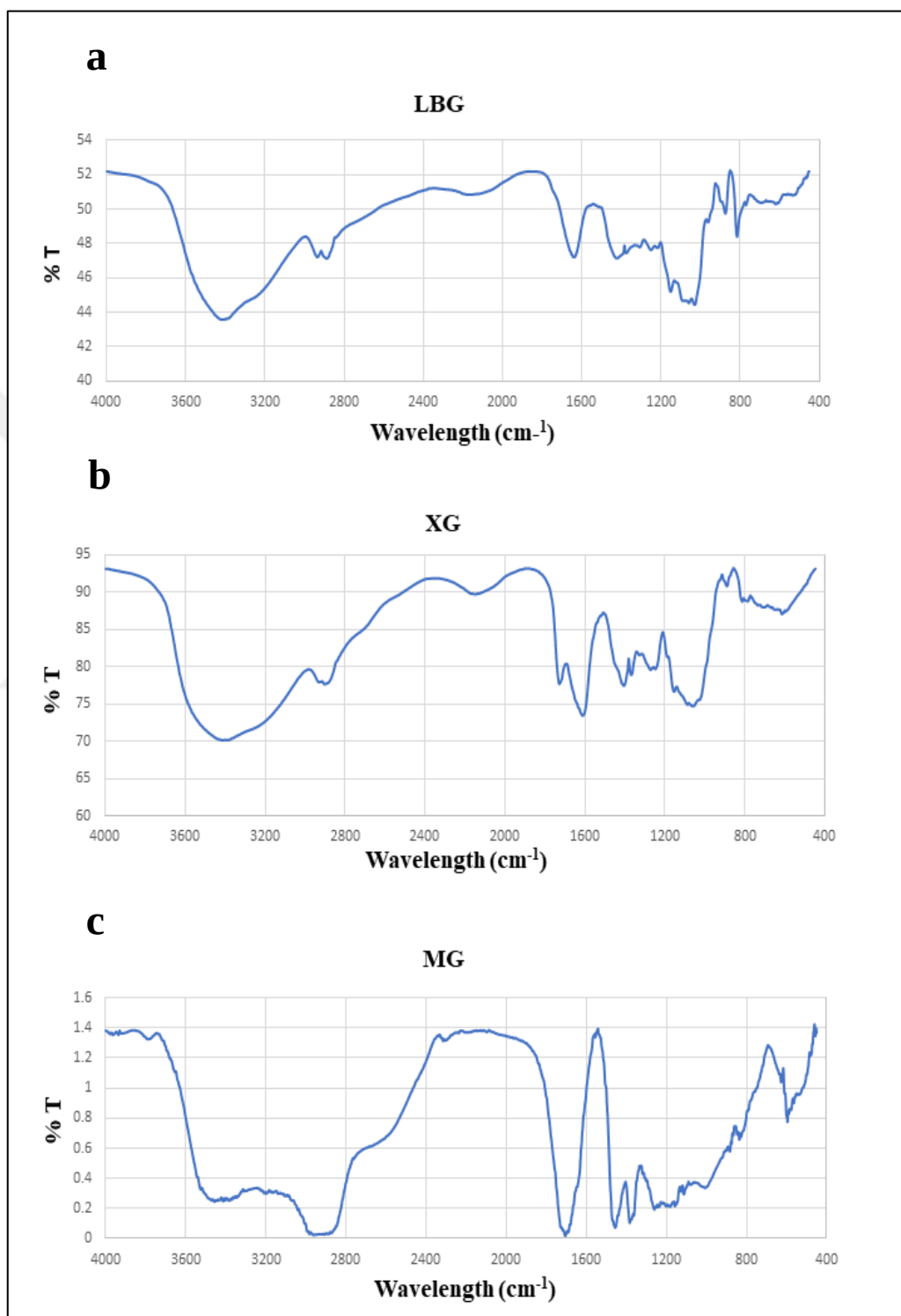
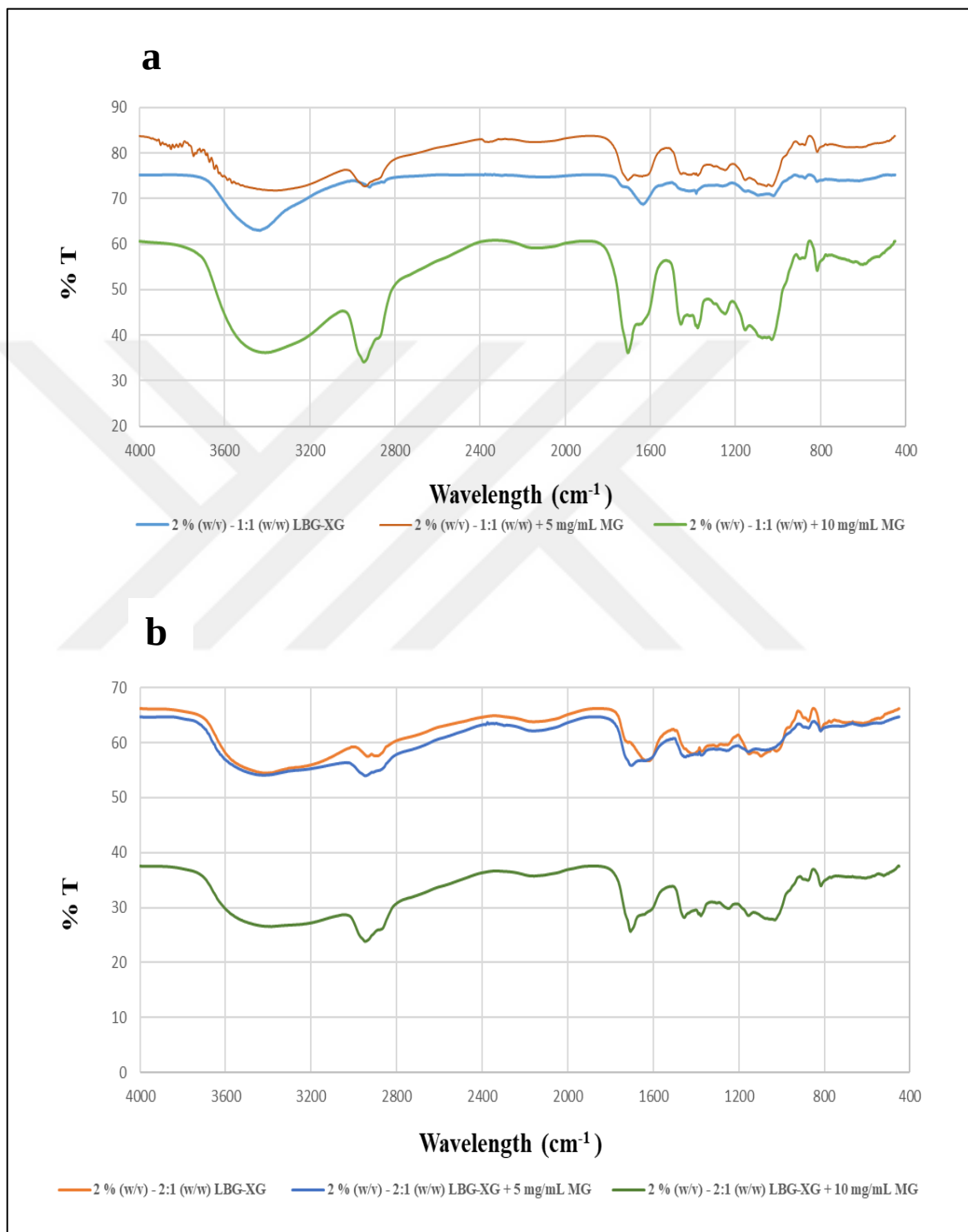
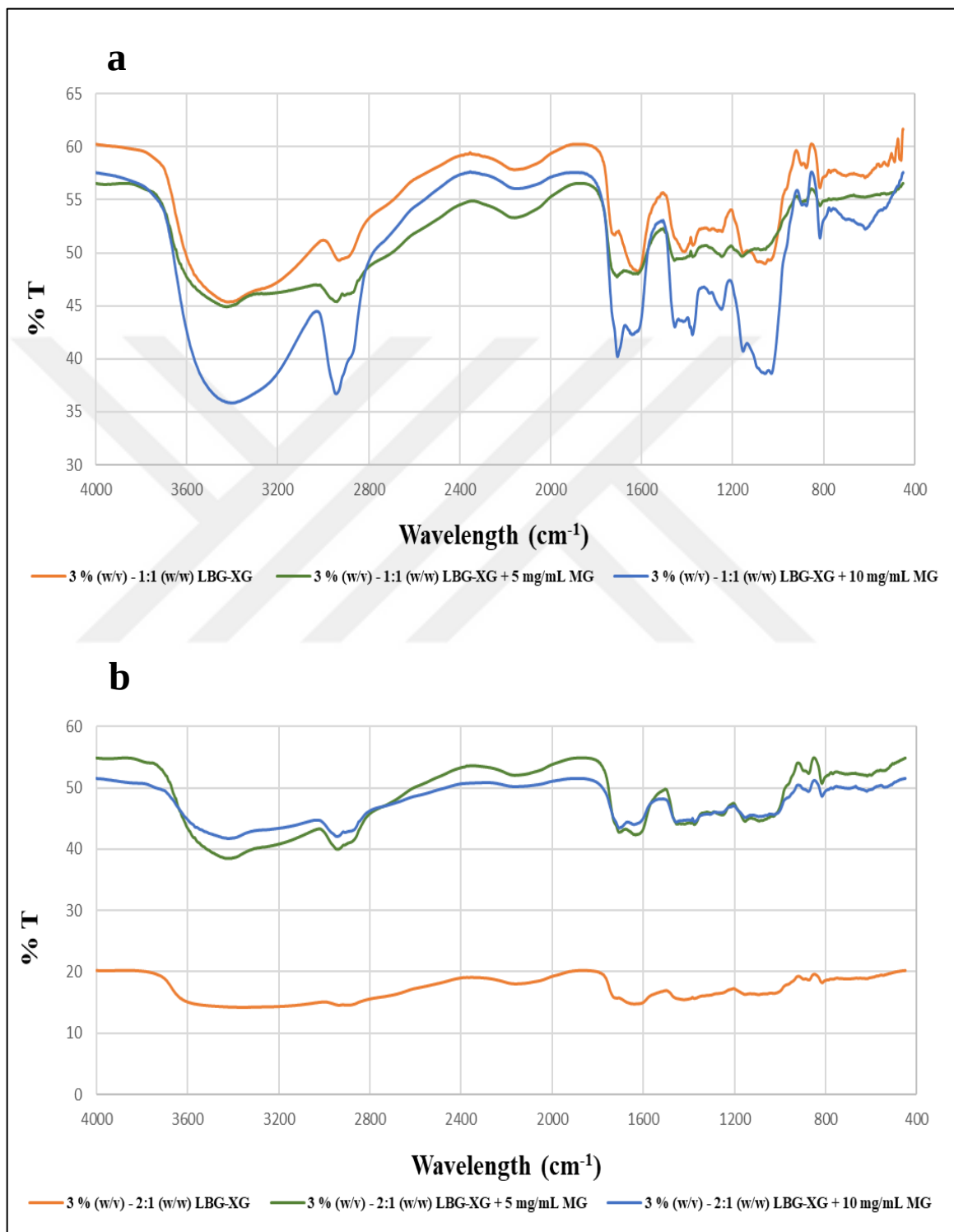


Figure 5.5. FT-IR spectra of (a) LBG, (b) XG, and (c) MG cryogels





5.1.5. DSC Analysis

DSC analysis was used for thermal characterization of cryogels. It measures the amount of heat required to increase the temperature during physical or chemical transition of the materials.

In Figure 5.8, LBG and XG had broad endothermic peak around 121 °C, and 119 °C, respectively. This peak is generally attributed to moisture loss [224], however, since the samples were lyophilized, the moisture content was negligible. Therefore, the first endothermic peak could be attributed to the presence of hydroxyl groups [225], or glass transition temperature (T_g). Furthermore, Upadhyay *et.al.* corresponded the first endothermic peak to a melting peak [226]. The exothermic peak of XG around 295 °C was associated with the degradation of the material [227]. MG had a small endothermic peak around 76 °C, which also became appeared in the MG containing samples (Figures 5.8 c, 5.9 a, b and 5.10 a, b). It might indicate T_g of MG. The peak becomes more distinctive in 10 mg/mL MG containing samples compared to 5 mg/mL MG containing ones. It did not exist in LBG, XG and LBG-XG cryogels. There was also a sharp exothermic peak around 379 °C in MG.

When MG was mixed with LBG and XG, the sharp peak around 379 °C disappeared (Figures 5.9 a, b and 5.10 a, b). The endothermic peaks from LBG and XG and the exothermic peak from XG were also preserved in blend cryogels (Figures 5.9 and 5.10).

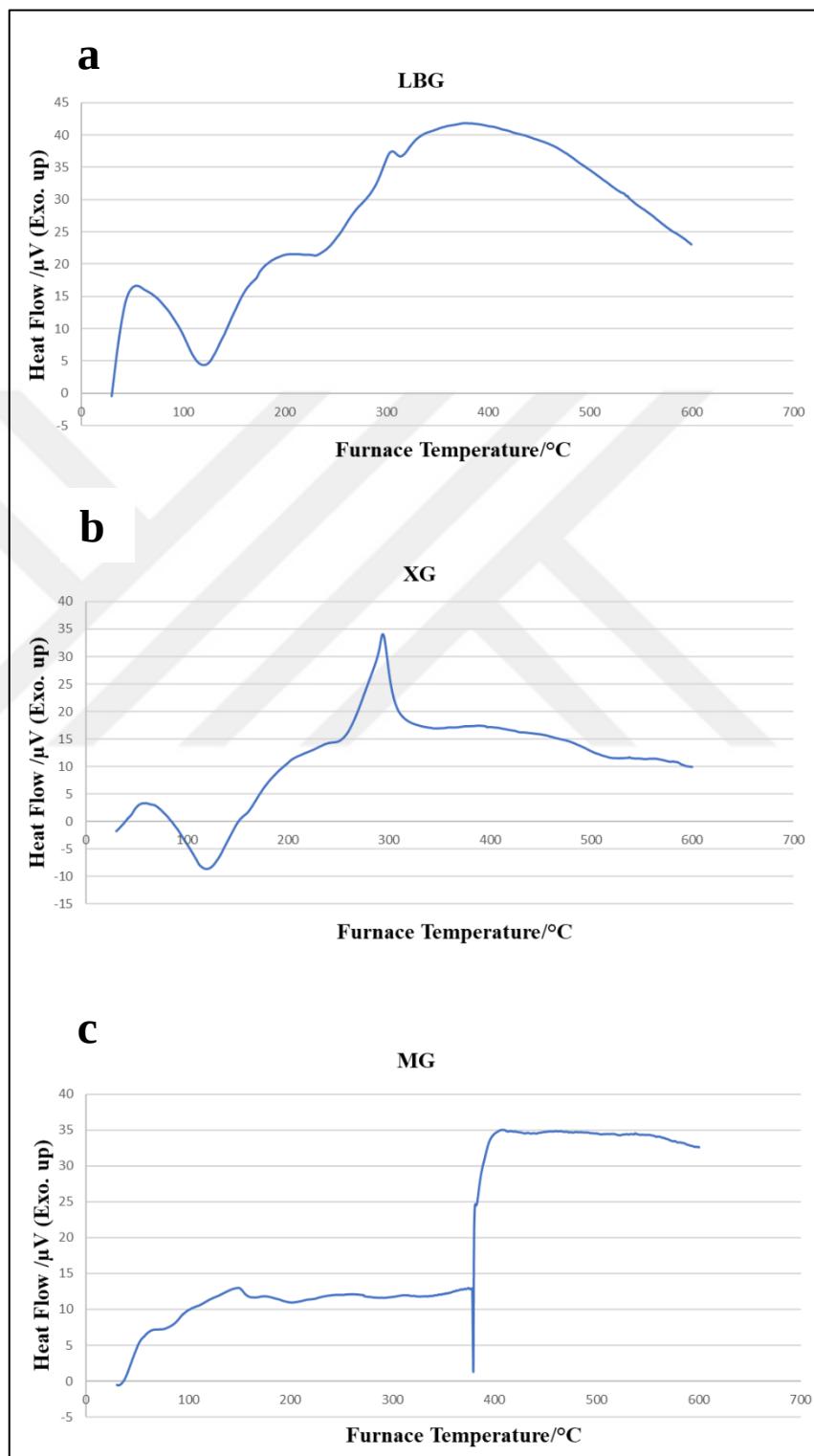


Figure 5.8. DSC thermograms of (a) LBG, (b) XG, and (c) MG

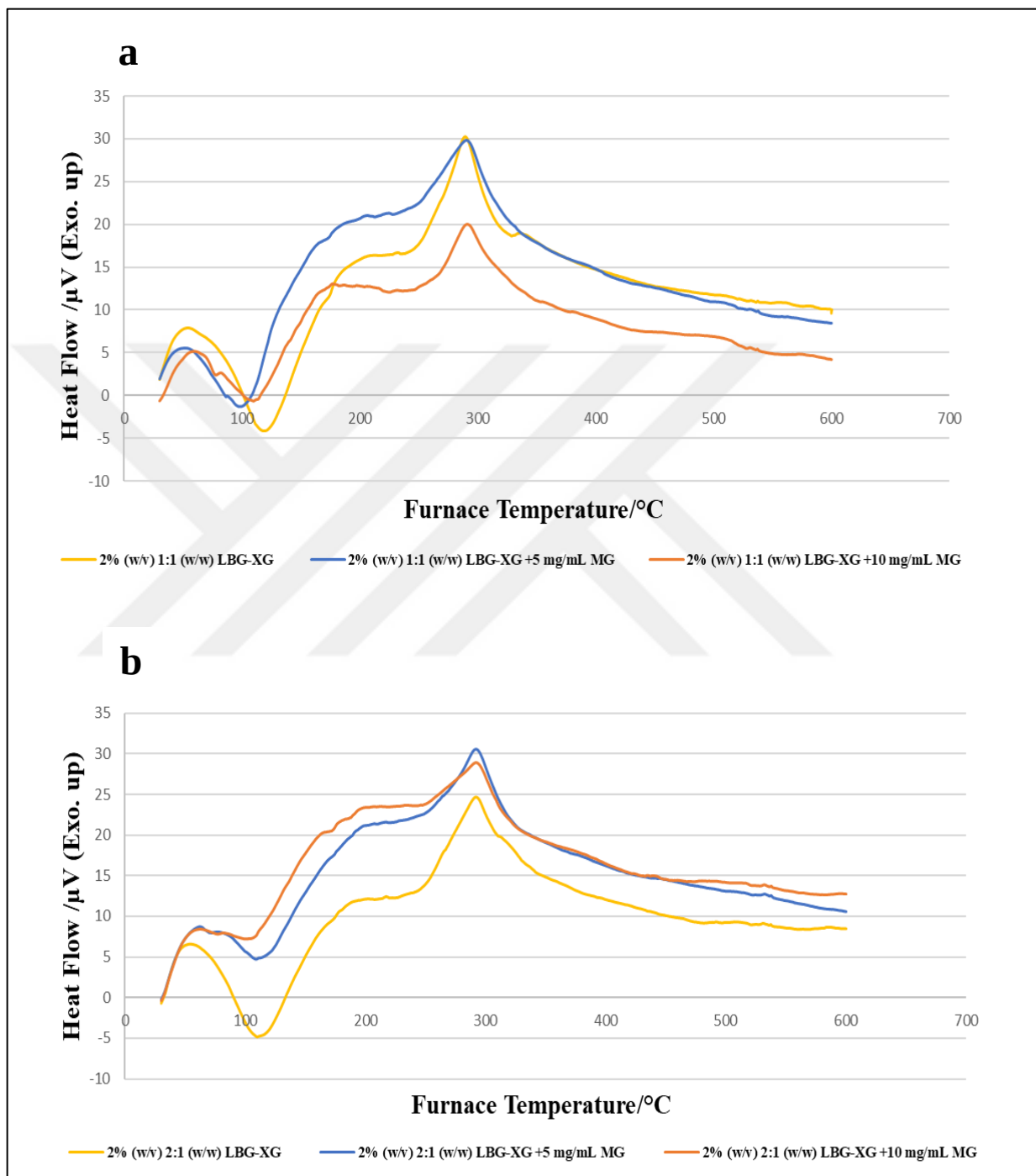


Figure 5.9. DSC thermogram of (a) 2 percent (w/v) – 1:1 (w/w) LBG-XG and LBG-XG-MG, and (b) 2 percent (w/v) – 2:1 (w/w) LBG-XG and LBG-XG-MG cryogels

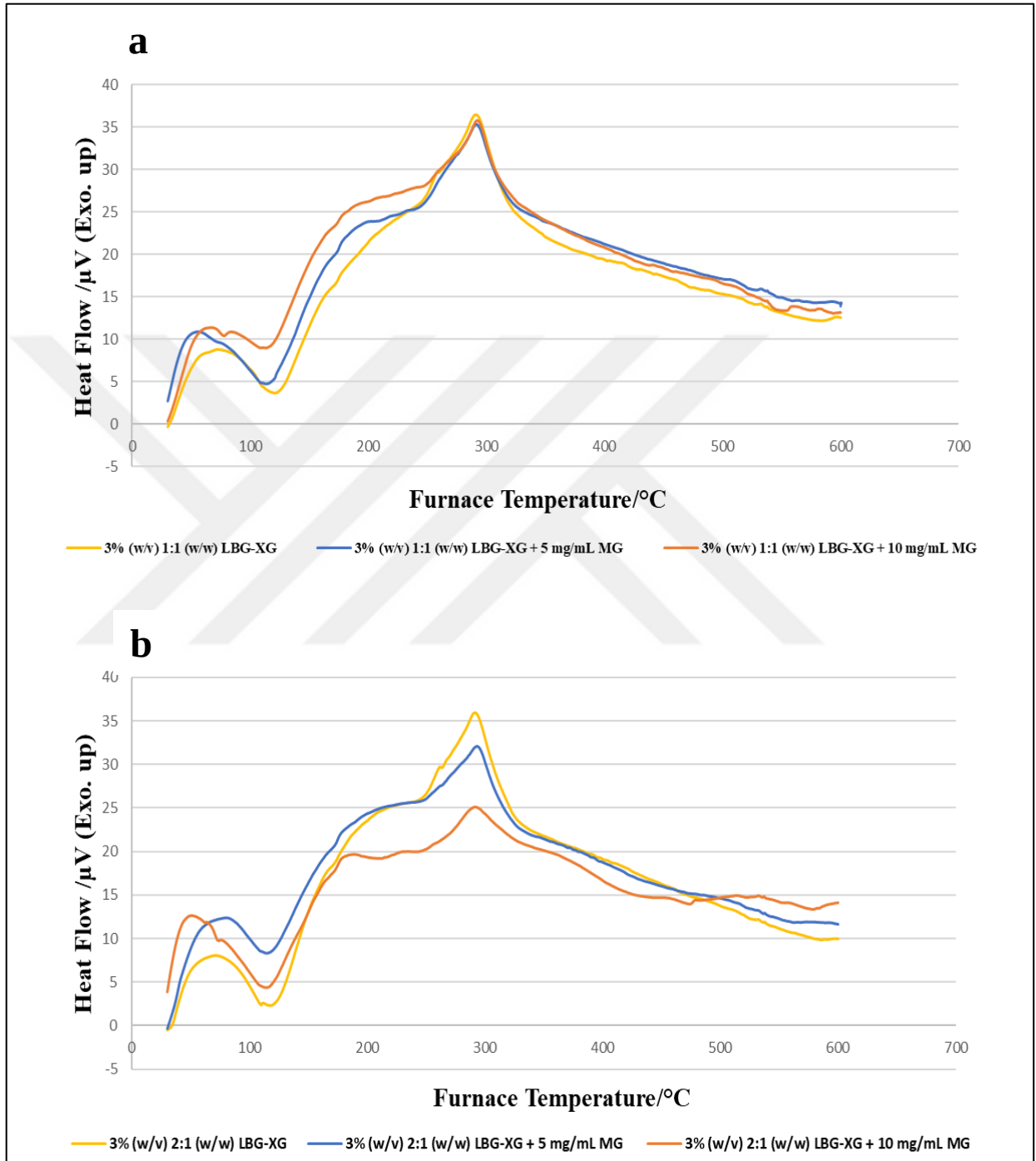


Figure 5.10. DSC thermogram of (a) 3 percent (w/v) – 1:1 (w/w) LBG-XG and LBG-XG-MG, and (b) 3 percent (w/v) – 2:1 (w/w) LBG-XG and LBG-XG-MG cryogels

5.1.6. Mechanical Characterizations

Mechanical characterizations were performed to determine the behavior of cryogels when compressive, tensile and shear forces were applied. In order to measure the stiffness of cryogels, Young's Modulus values were calculated upon subjection to different loads. Young's Modulus (E) is defined as the capability of a material to resist longitudinal forces and calculated as stress divided by strain.

$$E = \frac{F/A}{\Delta L/L_0} \quad (5.1)$$

Stress is the longitudinal force (F) applied to the crosssectional area (A) of the material, and strain is ratio of the amount of change in length (ΔL) to initial length (L_0) [228].

Tensile, compression and shear modulus values of samples were calculated for mechanical characterization of cryogels. Modulus values of cryogels were calculated for dry and wet states to examine the effect of swelling on mechanical properties. According to Figure 5.11, the highest and lowest tensile modulus values belonged to the 3 percent (w/v) – 2:1 (w/w) LBG-XG (9,41 MPa) and 2 percent (w/v) - 1:1(w/w) LBG-XG (1.88 MPa) respectively. There was a significant increase in the tensile modulus values of 2 percent (w/v) - 1:1(w/w) LBG-XG containing cryogels with the addition of MG. The tensile modulus values of MG containing 2 percent (w/v) - 1:1(w/w) LBG-XG and 2 percent (w/v) - 2:1 (w/w) LBG-XG cryogels were close to each other. Their modulus values were less than 3 percent (w/v) LBG-XG sample sets. Therefore, it can be concluded that the tensile modulus values increased with increasing concentration of LBG-XG in dry state. Furthermore, the positive effect of LBG on tensile modulus values could be deduced by the significant increase in tensile modulus values of 3 percent (w/v) – 2:1 (w/w) LBG-XG compared to 3 percent (w/v) – 1:1 (w/w) LBG-XG. Since the lowest modulus value belonged to the 2 percent (w/v) - 1:1(w/w) LBG-XG cryogels, it was decided to continue further experiments with the 2 percent (w/v) – 2:1 (w/w) LBG-XG and 3% (w/v) LBG-XG sample sets. Compressive modulus values of the remaining sample sets were calculated, and similar results with the tensile modulus values were obtained. There was a

significant increase in compressive modulus values of MG containing 3 percent (w/v) LBG-XG cryogels with increasing LBG concentration. Besides LBG concentration, the compressive modulus values of dry cryogels increased with increasing total polymer concentration. Shear modulus values also revealed that the high modulus values were directly proportional with increasing total polymer concentration and LBG amount. In 2 percent 2:1 (w/w) LBG-XG and 3 percent 2:1 (w/w) LBG-XG cryogels, shear modulus values were increased when 5 mg/mL MG added to the cryogels, however, when MG concentration was increased from 5 mg/mL to 10 mg/mL, the modulus values decreased. By the light of these results, it was observed that 2 percent (w/v) LBG-XG cryogel set had the lowest tensile, compressive and shear modulus values. The tensile, compressive, and shear modulus values of human articular cartilage are in the range of 9-18 MPa, 0.4-1.5 MPa, and 0.2-2.5 MPa respectively [229]. Dry cryogels' compressive and shear modulus values were in the modulus range of articular cartilage, but the tensile modulus values were close to the range. Therefore, it can be concluded that the cryogels met the desired modulus values of articular cartilage.

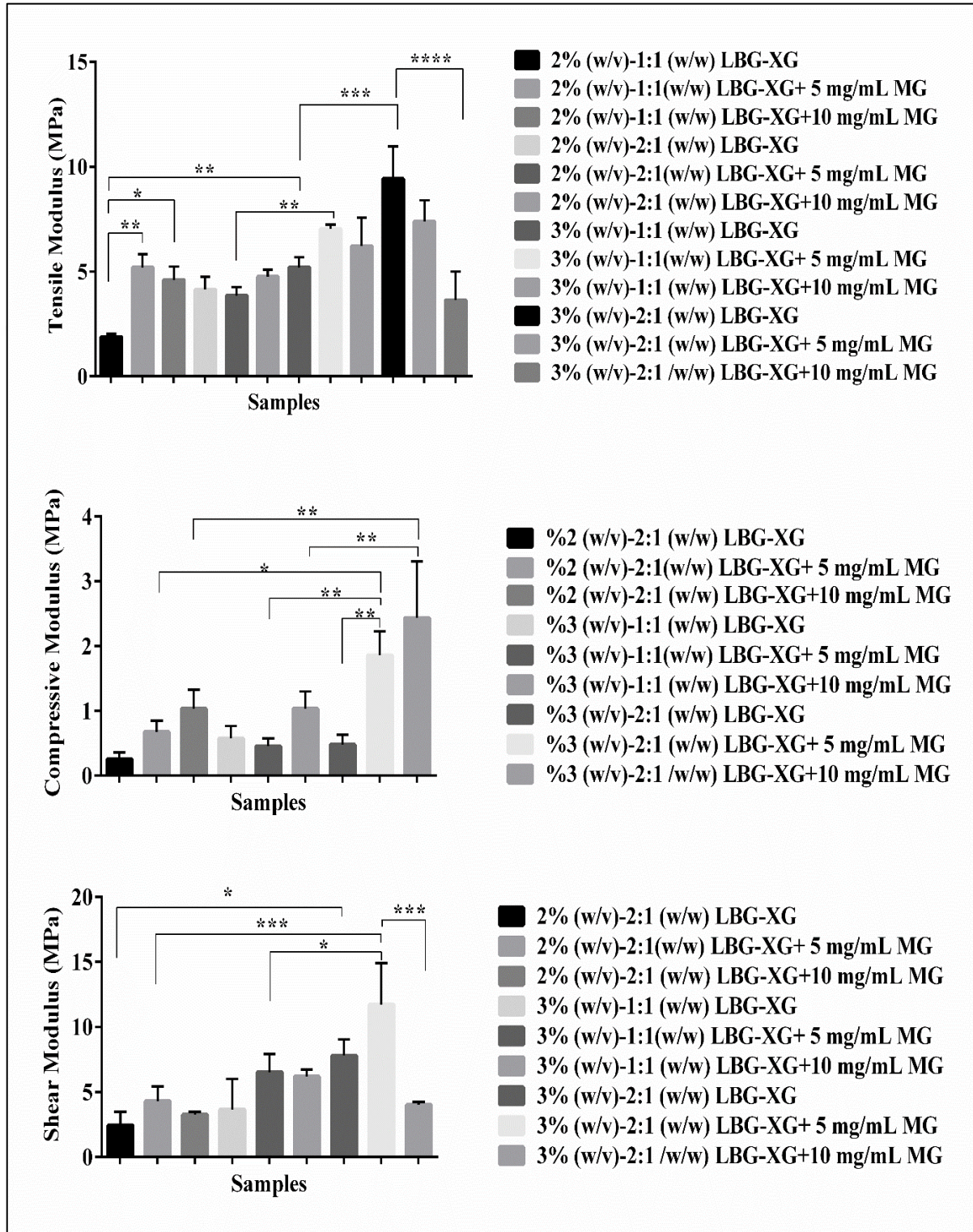


Figure 5.11. Young's Modulus values of dry cryogels (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

Figure 5.12 shows the modulus values of swollen (wet) cryogels. The lowest modulus values belonged to the cryogel set having 2 percent (w/v) LBG-XG concentration. The 3 percent (w/v) – 1:1 (w/w) LBG-XG cryogel had the highest compressive modulus value (393 kPa) among all samples. The MG containing 3 percent (w/v) – 1:1 (w/w) LBG-XG cryogels had less compressive modulus than the MG-free one. When tensile modulus values were examined, it was observed that 3 percent (w/v) – 1:1 (w/w) LBG-XG containing cryogel set had higher modulus values compared to the other sets. These values were also consistent with the compressive modulus values, however tensile modulus values of 3 percent (w/v) – 1:1 (w/w) LBG-XG increased with increasing MG concentration. Finally, the shear modulus values in Figure 5.12 showed that 3 percent (w/v) – 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels had the highest shear modulus, and 3 percent (w/v) – 1:1 (w/w) LBG-XG cryogel set containing 5 mg/mL MG, 10 mg/mL MG and MG-free samples had relatively high shear modulus compared to other sets. Unlike the modulus values of dry cryogels, compression, tensile and shear modulus values of wet cryogels increased with decreasing LBG concentration. The modulus values of wet cryogels were found consistent with the other polysaccharide hydrogels in the literature. Especially, the compressive modulus values of 3 percent (w/v) – 1:1 (w/w) LBG-XG containing cryogel set were higher than the compressive modulus values of the most polysaccharide scaffolds studied so far [230-234].

The difference in modulus values of dry and wet cryogels might be originated from the change in polymer chain organization during swelling [235]. Another reason of this situation might be the decrease in the number of polymer chains per volume in the swollen state [236].

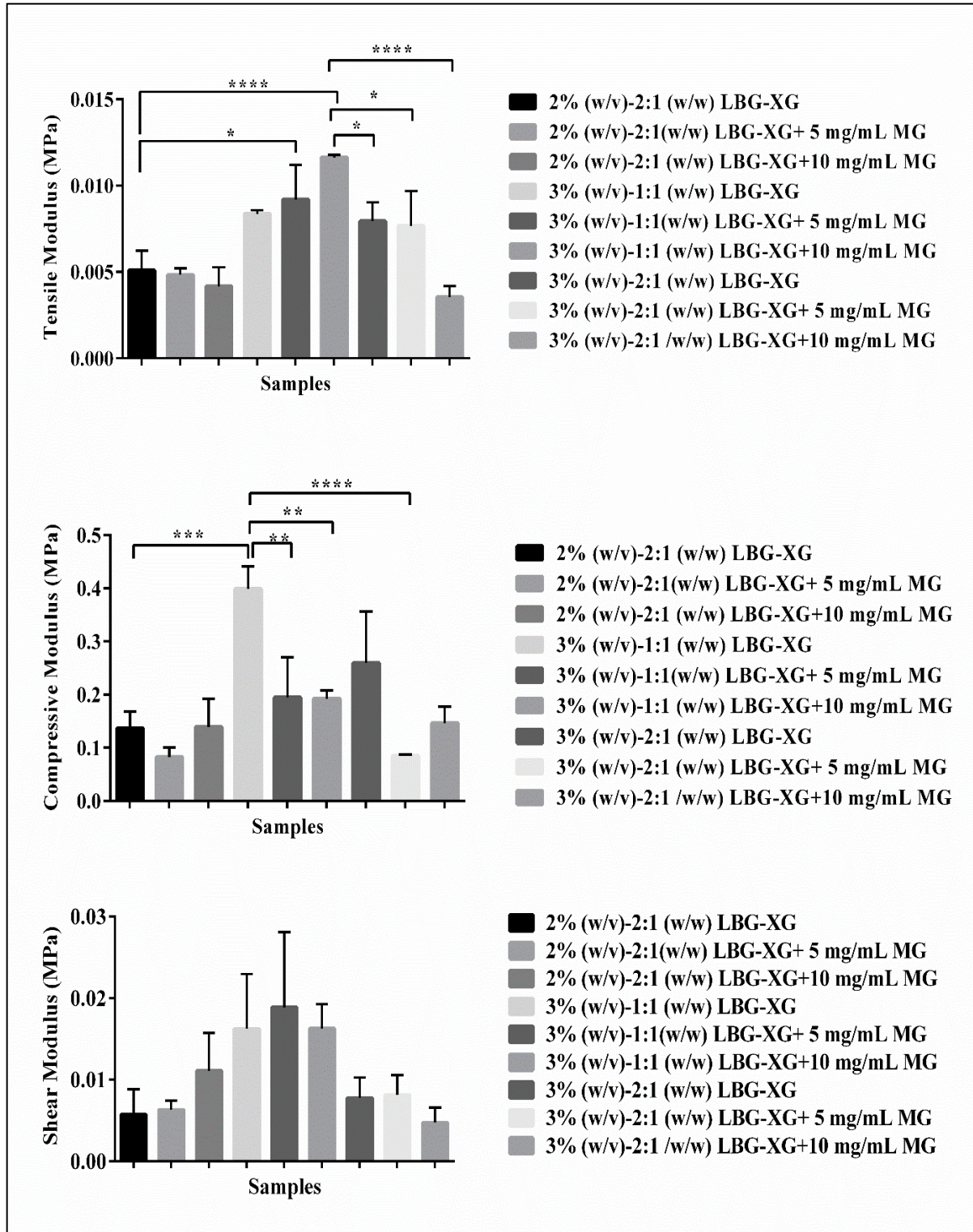


Figure 5.12. Young's Modulus values of wet cryogels (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

5.1.7. Release Studies of Kartogenin

Release studies were performed to designate release profile of KGN from the cryogels. The maximum absorbance wavelength value of the KGN was determined as 278 nm after wavelength scan. Different concentrations of KGN was loaded into the scaffolds and a calibration curve was plotted. Figures 5.13 and 5.14 show the percent cumulative release and cumulative amount of KGN released from the cryogels, respectively. At first three days period, cryogels released KGN faster than the other time intervals. The reason of this situation might be the release of KGN molecules remained in the surface of cryogels to the release solution. The release rate of KGN decreased after three days. Slower release rate indicates that the cryogels' physical integrity was maintained throughout 21 days. The cryogels having 2 percent (w/v) – 2:1 (w/w) LBG-XG, 3 percent (w/v) – 1:1 (w/w) LBG-XG, and 3 percent (w/v) – 2:1 (w/w) LBG-XG containing 5 mg/mL MG released more KGN (6.4, 5.5, 22.9 percent, respectively) compared to MG-free ones at the end of 21 days. The amount of KGN released from 2 percent (w/v) – 2:1 (w/w) LBG-XG, and 3 percent (w/v) – 2:1 (w/w) LBG-XG cryogels decreased when MG concentration increased from 5 mg/mL to 10 mg/mL. Conversely, concentration increase in MG resulted in an increase in KGN release in 3 percent (w/v) – 1:1 (w/w) LBG-XG cryogels from 39.9 percent to 44.6 percent. All cryogels, except 3 percent (w/v) – 2:1 (w/w) LBG-XG + 5mg/mL MG cryogel, released KGN within the range of 32-47 percent after 21 days of incubation. However, 3 percent (w/v) – 2:1 (w/w) LBG-XG + 5 mg/mL MG cryogel released nearly 53 percent of the loaded KGN at the end of day 7. The KGN delivery were performed via many different agents such as; injectable supramolecular hydrogels [237], collagen/cellulose nanocrystals/ultrasmall super-paramagnetic iron-oxide combination scaffolds [238], liposomes [239], dendrimers [240] and silk-derived nanosphere matrices [241] so far. In this study, the KGN was tried to be delivered via LBG-XG and LBG-XG-MG cryogels. According to these results, LBG-XG and LBG-XG-MG cryogels can be used for sustained release of KGN. The continuous release of KGN from cryogels induces its solubility and permeability. It also enhances KGN uptake by cells, thereby increasing its effectiveness for chondrogenic differentiation [242].

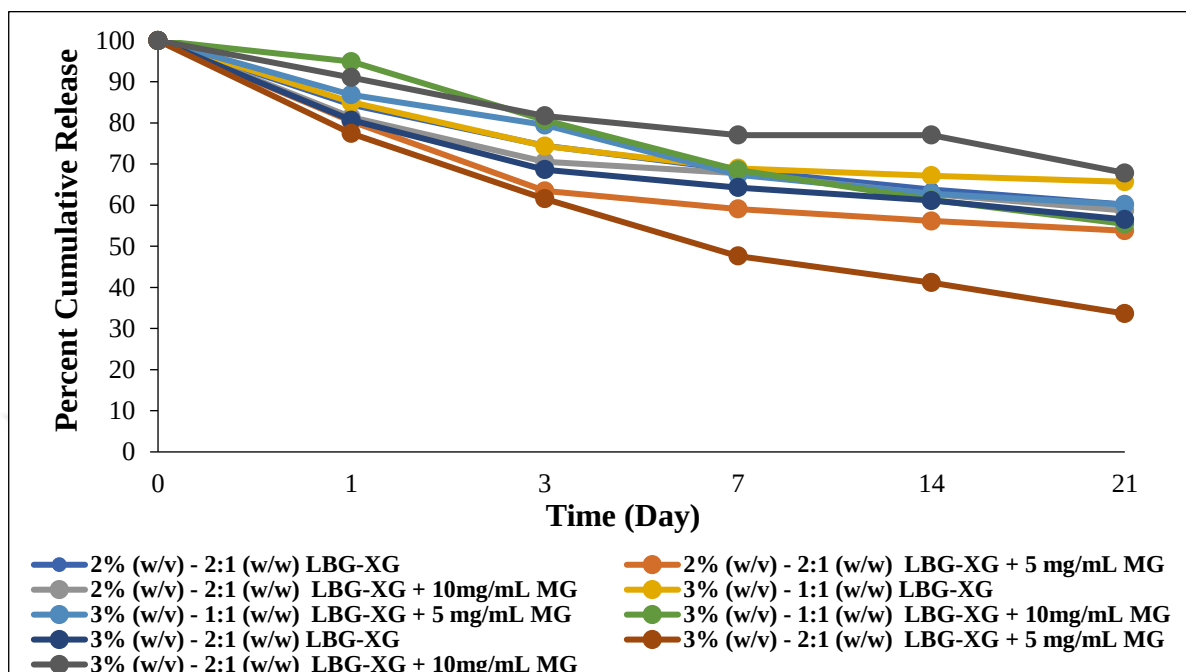


Figure 5.13. Percent cumulative KGN release from the cryogels

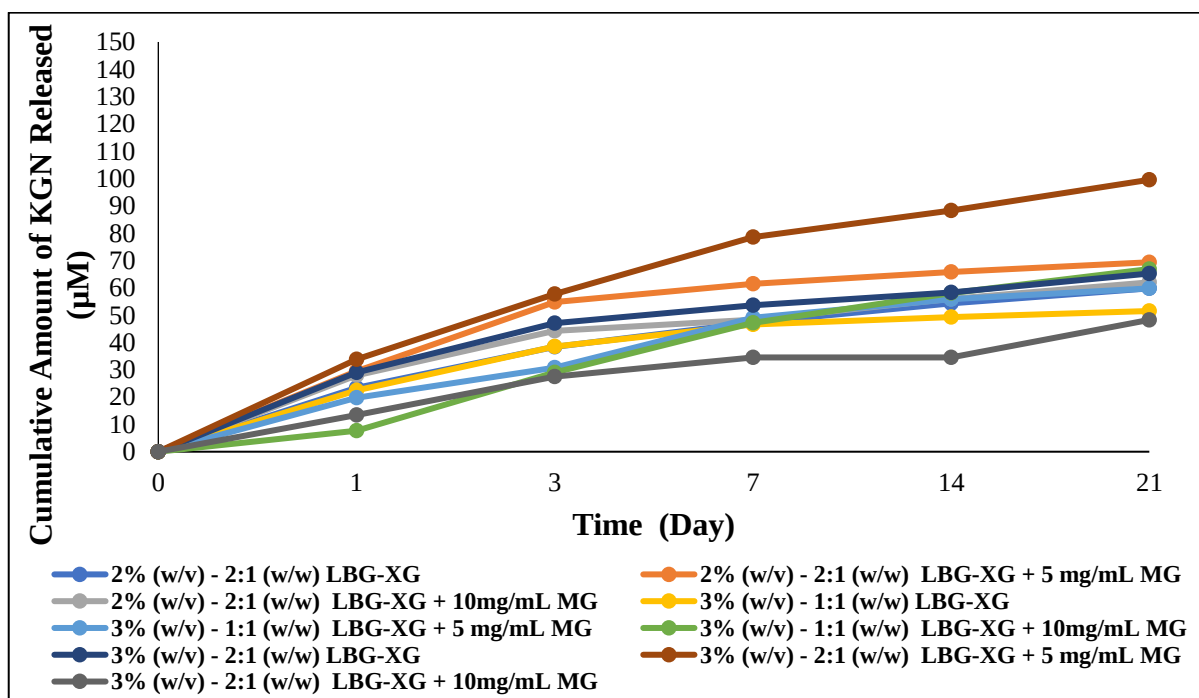


Figure 5.14. Cumulative amount of KGN released from the cryogels.

5.1.8. ^1H -NMR Spectroscopy

^1H -NMR spectroscopy is a molecular technique used for determination of chemical structure of a compound dissolved in a solvent, such as deuterated water (D_2O). This method characterizes molecules with respect to nuclei of ^1H [243, 244]. ^1H -NMR spectra of LBG, XG and MG are shown in Figure 5.15. The peak around 4.7 ppm indicated the presence of D_2O . When spectra of LBG and XG were investigated, many peaks between 3.4 ppm and 4.8 ppm were observed. In LBG, these peaks were associated with mannose/galactose, CH and CH_2 [245]. Characteristic peaks of XG were reported at 4.6 and 4.9 ppm that are corresponded to protons of hexose and pentose groups. Sugar groups in XG had peaks in the range of 3.0 – 5.5 ppm. The peaks at 1.4 and 2.1 ppm attributed to the pyruvate and acetate groups linked to mannose [221, 246-248]. MG is composed of many different compounds compared to LBG and XG. Three major groups found in the chemical structure of MG are; the polymeric region (cis-1,4-poly- β -myrcene), essential oil and triterpenes [249]. When the ^1H -NMR spectra of MG in the literature were examined, the distinguishing small peak around 8.2 ppm was not observed. As a result of a more comprehensive study, it was observed that the peak at 8.2 ppm fell into the region where the aromatic groups give peaks. However, the only group that caused a single peak instead of the clusters having many peaks often due to aromatic groups might belong to p-nitrophenol [250]. Since there are many different substances in materials isolated from plants such as MG, as mentioned above, it is thought that this peak might belong to one of them. The ^1H -NMR spectra of MG-free and MG containing LBG-XG cryogels (Figure 5.16 a, b, 5.17 a, b and 5.18 a, b), showed the characteristic peaks of LBG and XG. The MG preserved its specific peak at 8.2 ppm alongside peaks around 1 ppm when mixed with LBG-XG cryogels (Figures 5.16 b, 5.17 b, and 5.18 b). Total polymer concentration increase did not cause any dramatic change in the peaks between 3 and 5 ppm. However, when the spectrum of 3 percent (w/v) – 1:1 (w/w) LBG-XG and 3 percent (w/v) – 2:1 (w/w) LBG-XG compared, it was observed that the signal intensity of the peak around 1.8 ppm, which belonged XG, decreased. This situation might have originated from the LBG concentration, which could mask the signals coming from XG. The data obtained from ^1H -NMR spectroscopy indicated the successful blend formations of LBG-XG and LBG-XG-MG. These results also supported the FT-IR data.

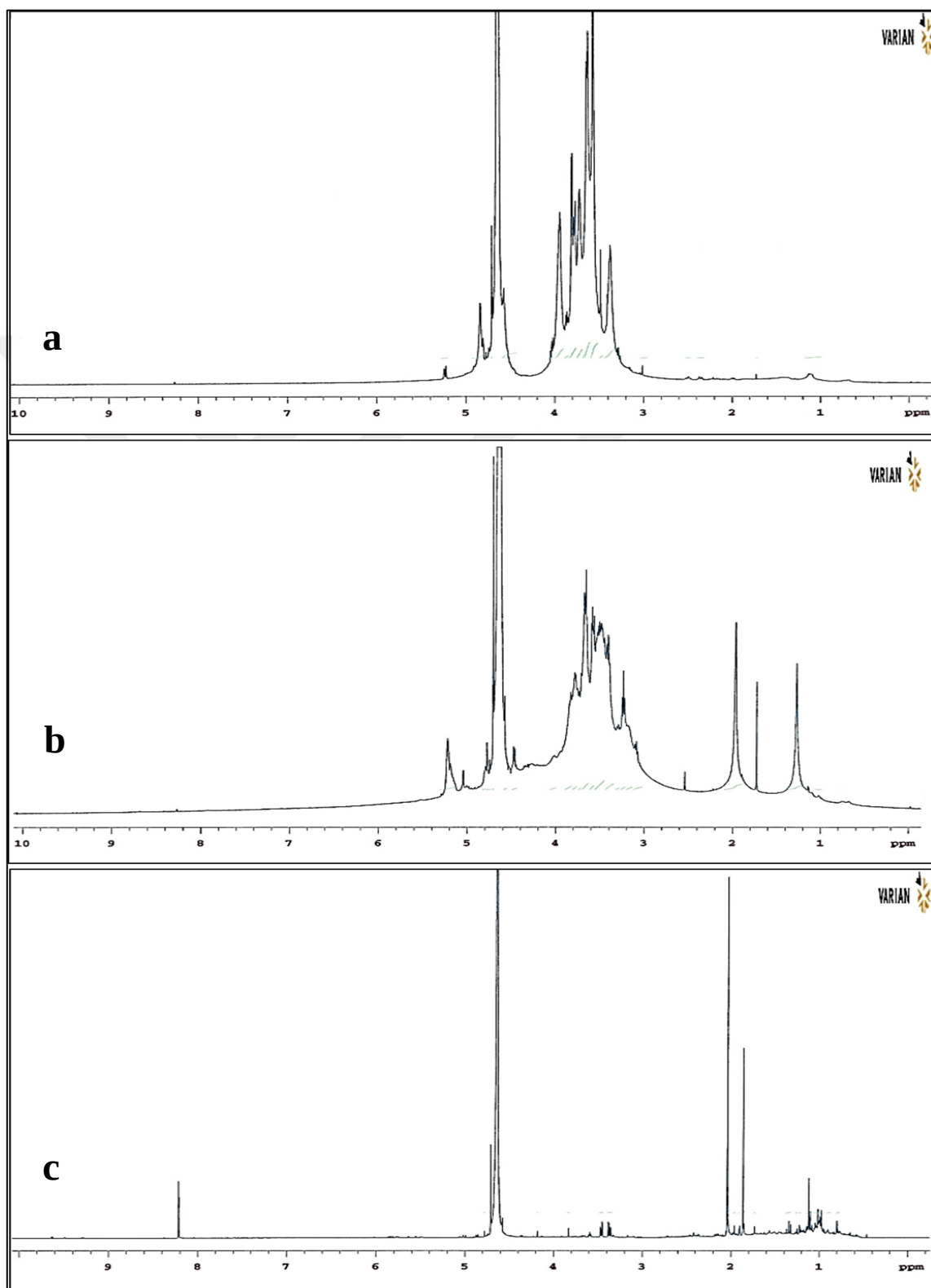


Figure 5.15. The ^1H -NMR spectra of (a) LBG (b) XG and (c) MG

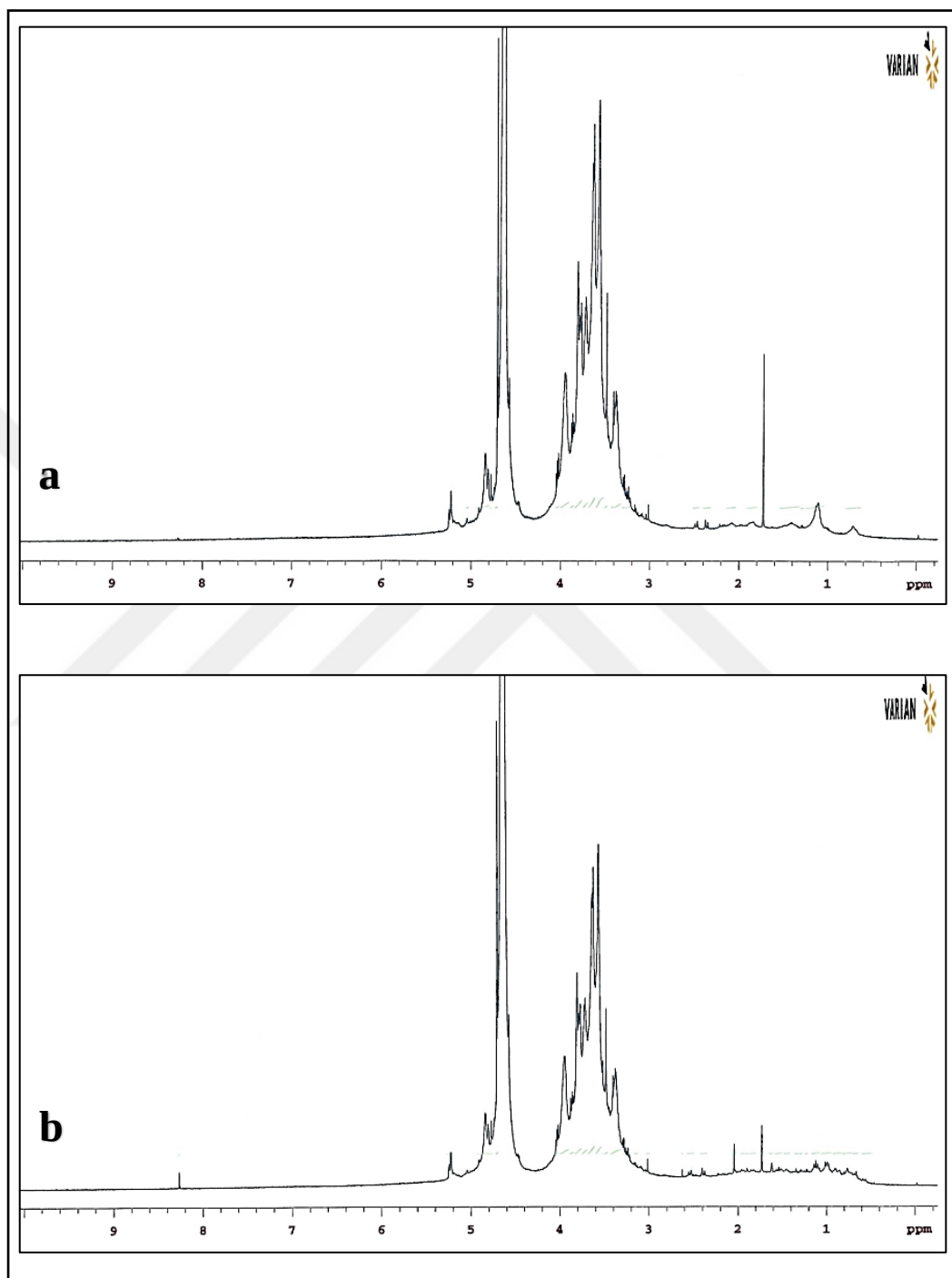


Figure 5.16. The ^1H -NMR spectra of (a) 2 percent (w/v) – 2:1 (w/w) LBG-XG (b) 2 percent (w/v) – 2:1 (w/w) LBG-XG + 5 mg/mL MG

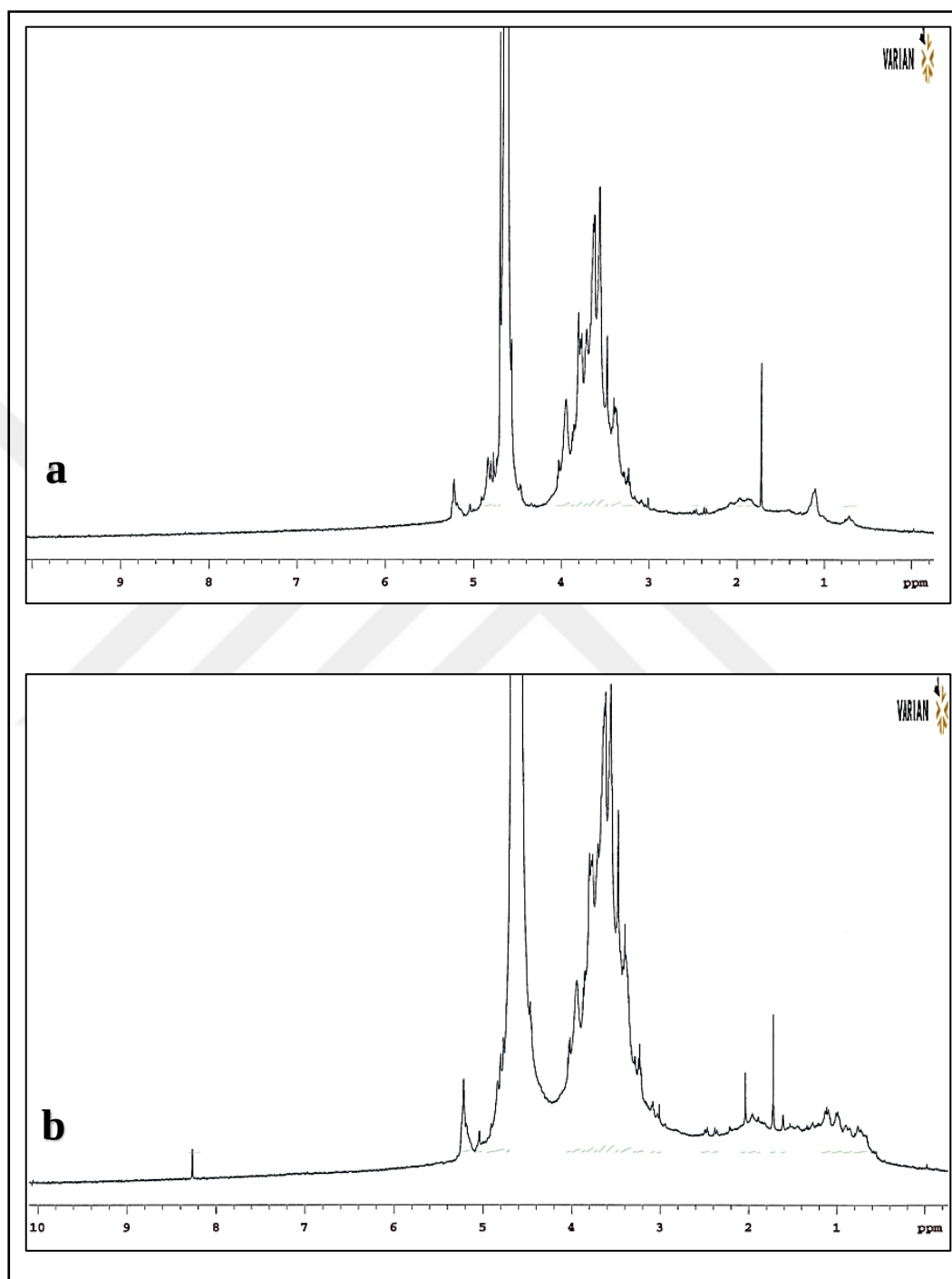


Figure 5.17. The ^1H -NMR spectra of (a) 3 percent (w/v) – 1:1 (w/w) LBG-XG (b) 3 percent (w/v) – 1:1 (w/w) LBG-XG + 5 mg/mL MG

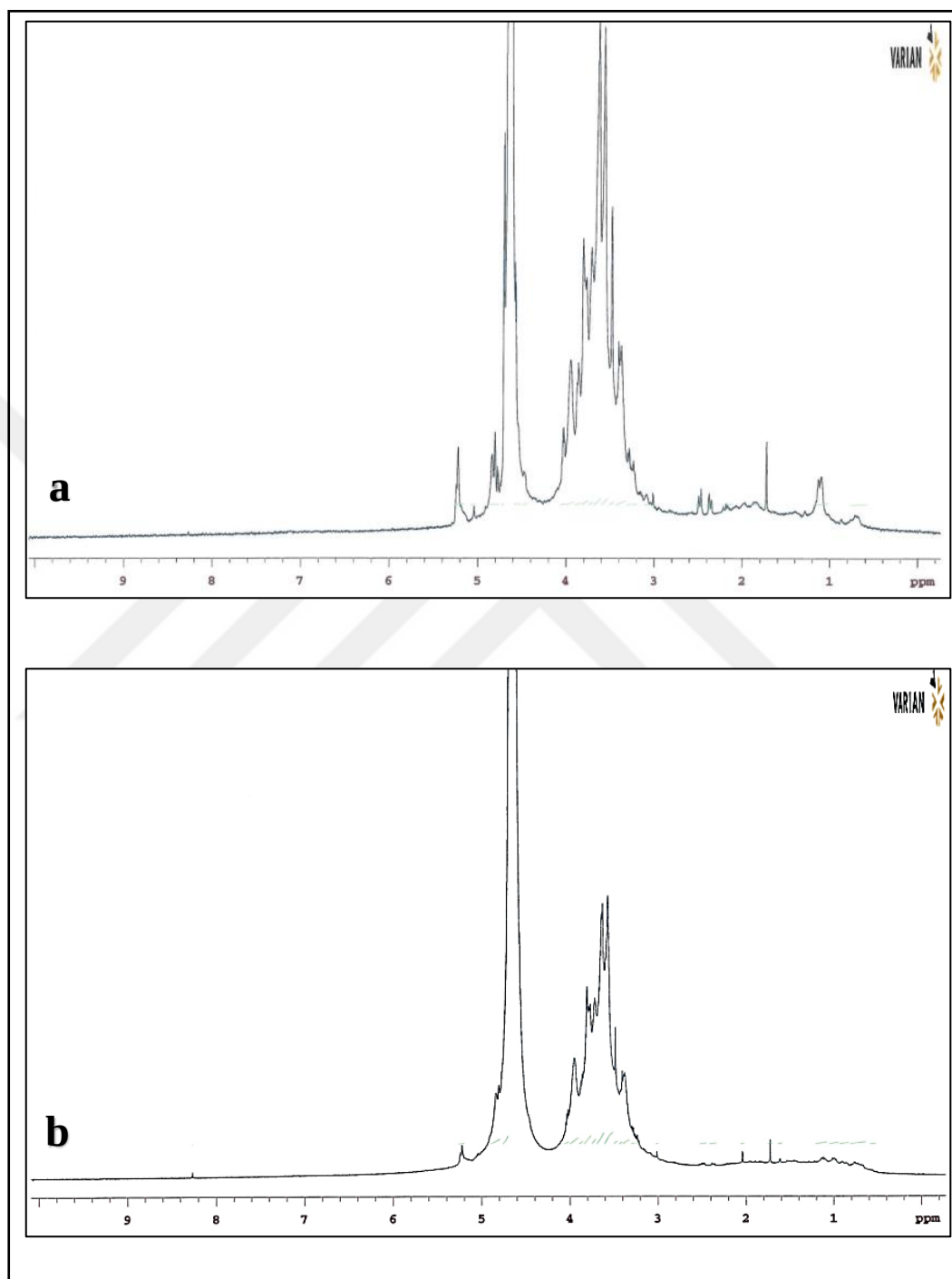


Figure 5.18. The ^1H -NMR spectra of (a) 3 percent (w/v) – 2:1 (w/w) LBG-XG (b) 3 percent (w/v) – 2:1 (w/w) LBG-XG + 5 mg/mL MG

5.2. IN VITRO TESTING OF CELLS AND CRYOGELS

5.2.1. Isolation and Characterization of RBM-MSCs

The MSCs are plastic-adherent multilineage cells mainly able to differentiate into chondrogenic, osteogenic, adipogenic and myogenic cells under cell culture conditions. They should express specific cell surface markers such as CD 90 and CD 29 and should not express hematopoietic markers CD 45 and CD 34 [251]. The flow cytometry analysis was performed to determine whether isolated cells from rat bone marrow were mesenchymal stem cells or not. For this purpose, expression of CD 90, CD29, CD34 and CD 45 were analyzed via flow cytometry. According to Figure 5.19, cells isolated from bone marrow of Sprague Dawley rats were able to express CD 29 and CD 90 cell surface markers with the percentages of 96.05 and 99.78 respectively. Most of the cells did not express hematopoietic stem cell markers CD 45 (8.18 percent) and CD 34 (9.11 percent), as it was expected.

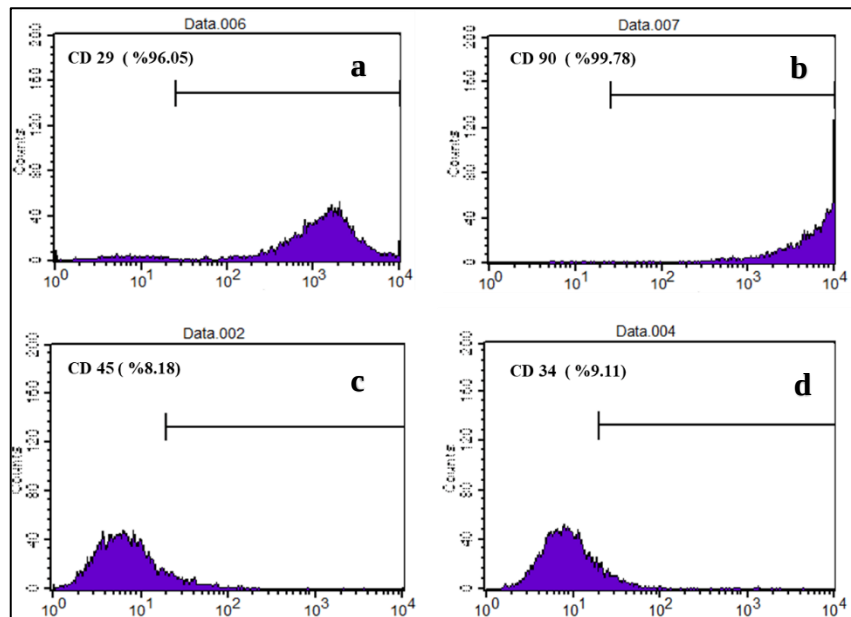


Figure 5.19. Flow cytometry results of RBM-MSCs expressing (a) CD 29, (b) CD90, (c) CD45, (d) CD34

Alcian Blue staining was performed to determine chondrogenic differentiation of RBM-MSCs. The dye stains acid mucins, proteoglycans and hyaluronic acid blue; neutral mucins, glycogens and glycoproteins red and the mixture of both purple. The nuclei of the cells are stained pale blue. Figure 5.20 a-f shows the Alcian Blue staining of the cells at days 7, 14 and 21 after the chondrogenic induction. According to the staining results, cells were differentiated towards chondrocytes. They became smaller and more rounded on 21st day of chondrogenic induction. While the extracellular matrices of the cells incubated in chondrogenic differentiation media stained blue and purple, the control groups were stained pale blue or not stained at all. This situation indicates that the cells were differentiated into chondrocytes.

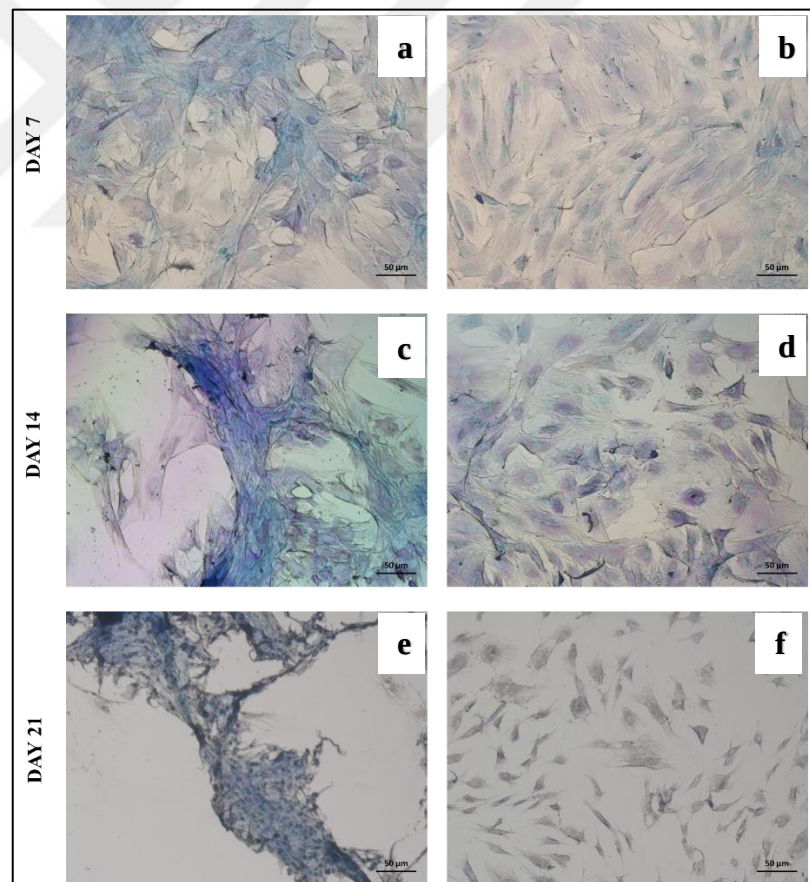


Figure 5.20. Alcian Blue stainings upon chondrogenic induction of RBM-MSCs (a, c, e) and untreated RBM-MSCs (b, d, f) at days 7 (A, B), 14 (c, d), and 21 (e, f) (20 X magnification-scale bars:50 µM)

In order to prove multilineage differentiation capacity of cells, Oil Red O staining was performed. Oil Red O is a fat soluble non-fluorescent dye that stains neutral lipids (triglycerides and diacylglycerols) and cholesterol esters [252]. The adipogenic differentiation was observed after 7, 14 and 21 days following adipogenic induction. The cells became more rounded like adipocytes and they produced lipid droplets which were stained red via Oil Red O stain (Figure 5.21 a-f). The size of lipid droplets became larger from day 7 to day 21.

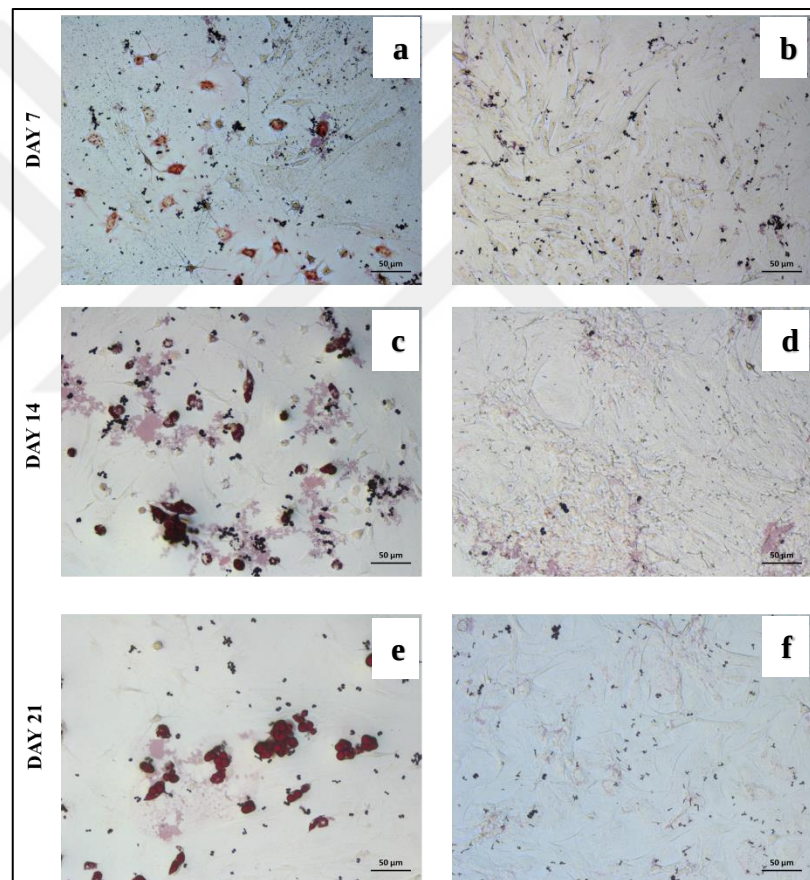


Figure 5.21. Oil Red O stainings upon adipogenic induction of RBM-MSCs (a, c, e) and untreated RBM-MSCs (b, d, f) at days 7 (a, b), 14 (c, d), and 21 (e, f) (20 X magnification-scale bars:50 μ M)

Thirdly, osteogenic induction was carried out with cells isolated from bone marrow of rats. Alizarin Red stain solution was used to visualize calcium deposits produced by cells. Alizarin

red S (1,2-dihydroxy-9,10-anthraquinonesulfonic acid sodium salt) is an anthraquinone dye which stains calcium generated by cells. Following osteogenic induction, cells differentiated to osteocytes and started to produce calcium deposits (Figure 5.22 a-f). The calcium deposition was increased from day 7 to day 21 in cells after osteogenic induction. The Alcian Blue, Oil Red O, and Alizarin Red staining results indicate that rat bone marrow cells were differentiated towards three major lineages, therefore the mesenchymal properties of these cells were proven by these assays.

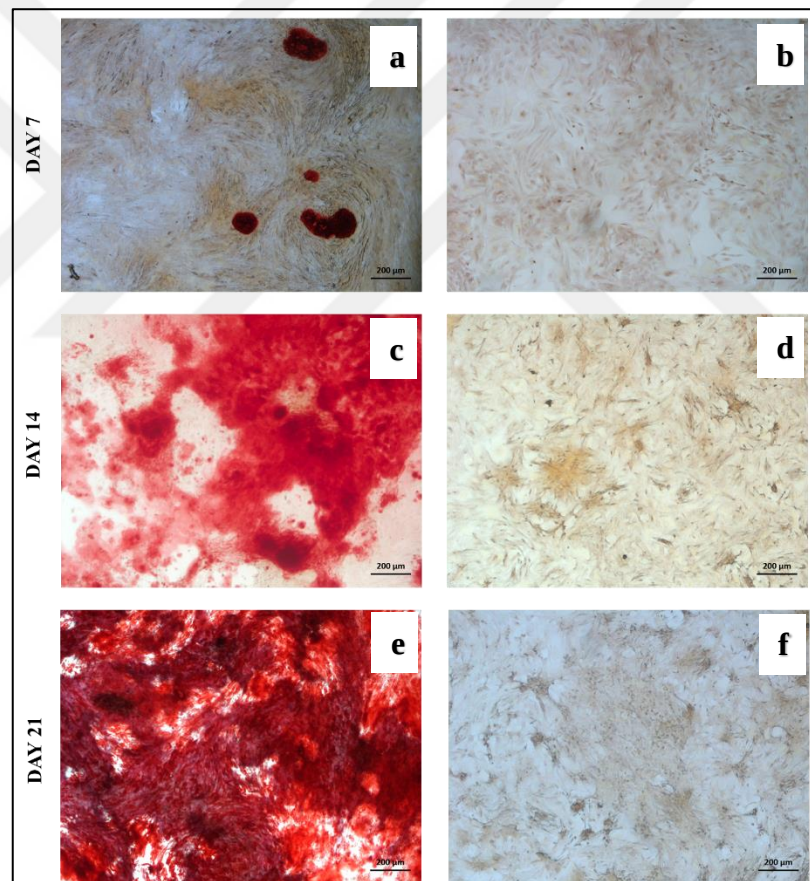


Figure 5.22. Alizarin Red stainings upon osteogenic induction of RBM-MSCs (a, c, e) and untreated RBM-MSCs (b, d, f) at days 7 (a, b), 14 (c, d), and 21 (e, f) (5 X magnification-scale bars:200 µM)

5.2.2. Effect of KGN on Chondrogenic Differentiation

The effect of different concentrations of KGN on RBM-MSCs was examined throughout 21 days. Besides chondrogenic stimulator effect of KGN, it was proven that KGN also induces the proliferation of cartilage stem/progenitor cells [253]. When chondrogenic differentiation of cells incubated in KGN containing growth medium (KGN+GM) and KGN containing chondrogenic medium (KGN + CM) were compared (Figures 5.23, 5.24, and 5.25), it was observed that, in KGN medium the cells were proliferated aside from chondrogenic stimulation. Figure 5.23 showed the images of the cells incubated in CM. It was observed that cells incubated in CM produced less cartilage specific ECM molecule than the cells incubated in KGN + GM and KGN + CM media. On the other hand, upon induction of cells with KGN + CM, the number of cells did not increase as much as the number of cells incubated in KGN + GM. The chondrogenic differentiation was also observed in cells treated with KGN + GM. The production of cartilage specific ECM molecules was increased from day 7 to day 14. The cells treated with 150 nM KGN + GM and KGN + CM medium produced more cartilage specific ECM molecules than cells treated with media containing 100 nM and 150 Nm KGN, especially on day 14. From day 14 to day 21, cells continued to produce cartilage specific molecules, however, cells treated with 150 nM KGN produced more proteoglycans and hyaluronic acid than glycoproteins; which was deduced from the intensity of blue stains on 21st day of chondrogenic induction. The cells induced with 150 and 200 nM KGN containing growth media produced matrix stained with purple on day 21. The color purple indicates the production of proteoglycans, hyaluronic acid and glycoproteins at the same time. The positive effect of KGN on chondrogenic differentiation of RBM-MSCs was proven with respect to these results. The 150 nM KGN containing medium was selected for the purpose of chondrogenic induction of cells for further experiments, since cells incubated in this medium synthesized more cartilage specific matrix molecules than others.

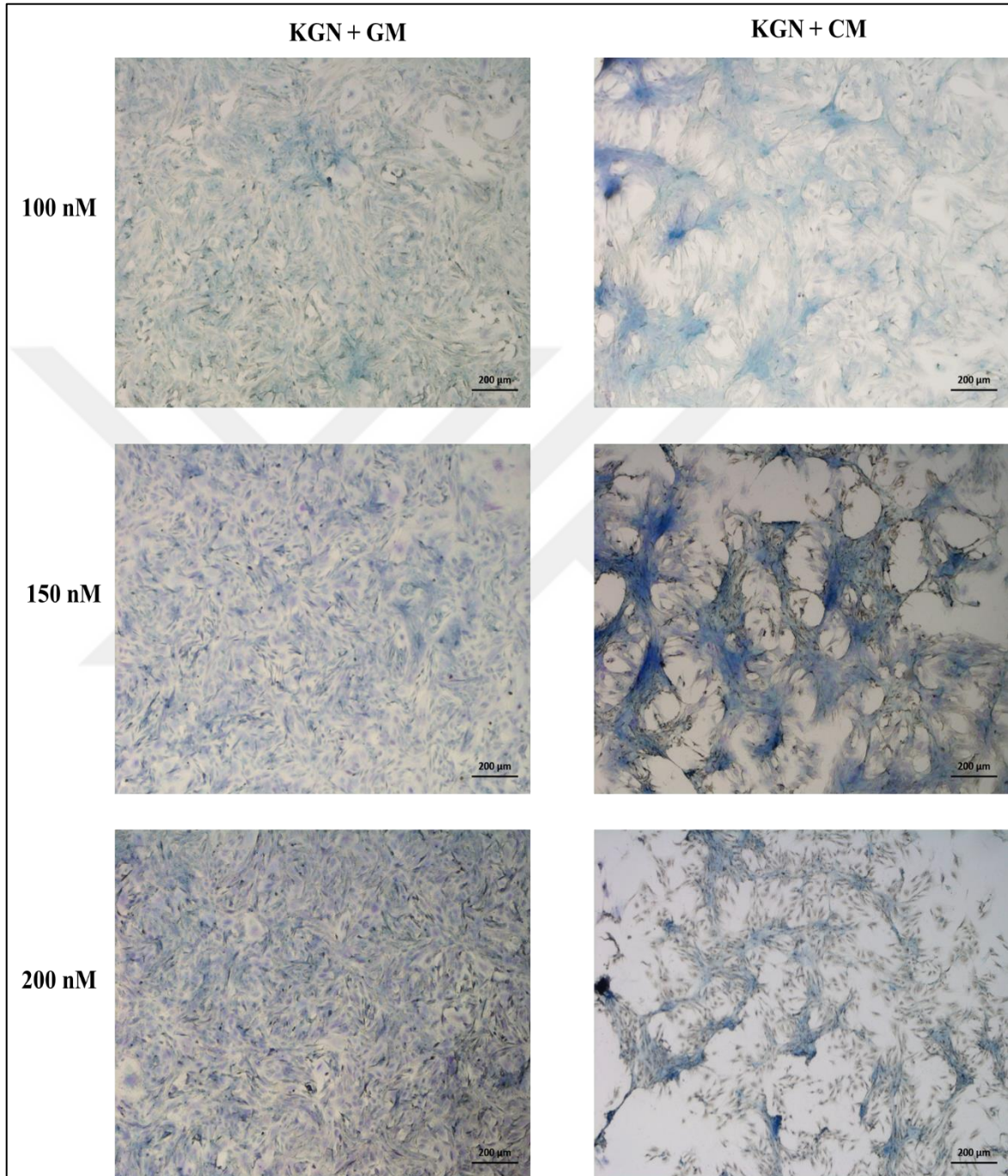


Figure 5.23. Alcian Blue stainings of RBM-MSCs upon chondrogenic induction in either KGN+GM or KGN+ CM with different concentrations of KGN at day 7 (20 X magnification- scale bars:200 μM)

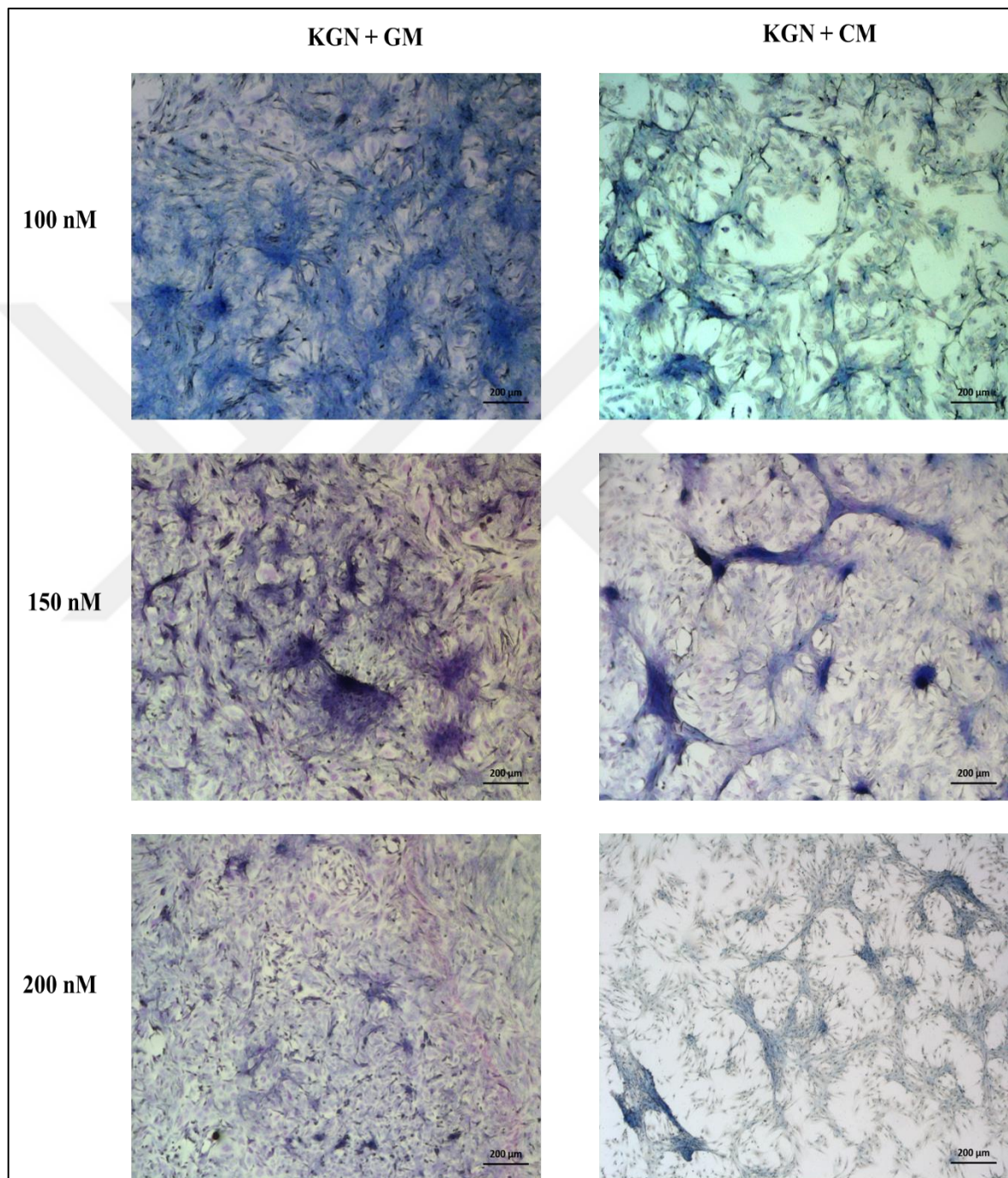


Figure 5.24. Alcian Blue stainings of RBM-MSCs upon chondrogenic induction induction in either KGN+GM or KGN+ CM with different concentrations of KGN at day 14 (5X magnification- scale bars:200 μM)

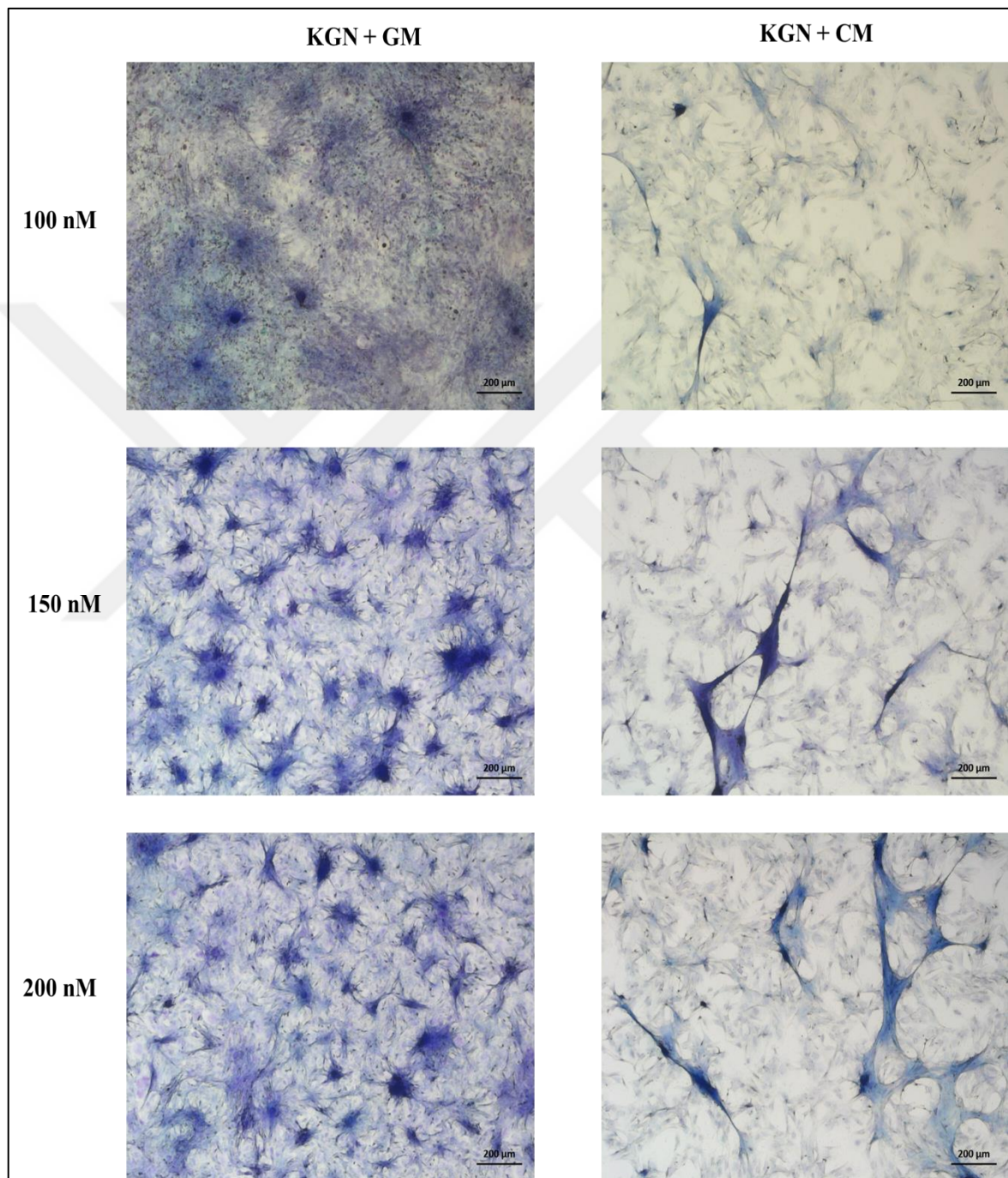


Figure 5.25. Alcian Blue stainings of RBM-MSCs upon chondrogenic induction induction in either KGN+GM or KGN+ CM with different concentrations of KGN at day 21 (5X magnification scale bars:200 μM)

5.2.3. Cell Viability Analysis

5.2.3.1. CellTiter 96® AQueous One Solution Cell Proliferation Assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric procedure for determining proliferation and viability of cells. The reagent consists of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The viable cells reduce the MTS tetrazolium reagent to formazan products, which is readily soluble in cell culture media. The formazan product has maximum absorbance at 490 nm [254]. Higher absorbance values signify an increase in cell proliferation.

Figure 5.26 shows the results of cell viability assay applied to RBM-MSC-laden cryogels. The initial cell attachment in 2 percent (w/v) and 3 percent (w/v) LBG-XG cryogels was higher than in MG-free cryogels. The absorbance values increased in 2 percent (w/v) - 2:1 (w/w) LBG-XG + 10 mg/mL MG, 3 percent (w/v) - 2:1 (w/w) LBG-XG + 5 mg/mL MG, and 3 percent (w/v) - 2:1 (w/w) LBG-XG + 10 mg/mL MG cryogels from day 3 to day 21. Cryogels having concentrations of 2 percent (w/v) - 2:1 (w/w), 2 percent (w/v) - 2:1 (w/w) + 5 mg/mL, 3 percent (w/v) - 1:1 (w/w), and 3 percent (w/v) - 2:1 (w/w) LBG-XG had an increase in the absorbance values from day 3 to day 7. However, after day 7, the absorbance values of these cryogels started to decrease. The absorbance values of 3 percent (w/v) - 1:1 (w/w) + 5 mg/mL and 3 percent (w/v) - 1:1 (w/w) + 10 mg/mL cryogels first decreased from day 3 to 7, and then started to increase from day 7 to 14. Cryogels having 3 percent (w/v) - 1:1 (w/w) LBG-XG concentration had significantly less absorbance value than the one in 2 percent (w/v) - 2:1 (w/w) LBG-XG on day 7. Furthermore, MG containing scaffolds had less absorbance values than the MG-free ones. In literature, there were many studies investigating the relationship of hydrogels and mesenchymal stem cells. Those studies revealed that the percent cell viabilities or the absorbance values were increased within first 7 days, which is consistent with our data [255-257]. Besides these studies, there were also some other articles showing the percent viability of cells remain constant through the cultivation periods [258-262]. Apart from these, Jooybar *et*

al. showed that the metabolic assay absorbance values obtained from MSC-laden hydrogels were increased from day 1 to 3, and afterwards they started to decrease, which is also similar with our results [263]. Regarding these results and studies performed previously, it can be concluded that RBM-MSCs were attached and proliferated in all cryogels. They provided a suitable environment for cells to live in.

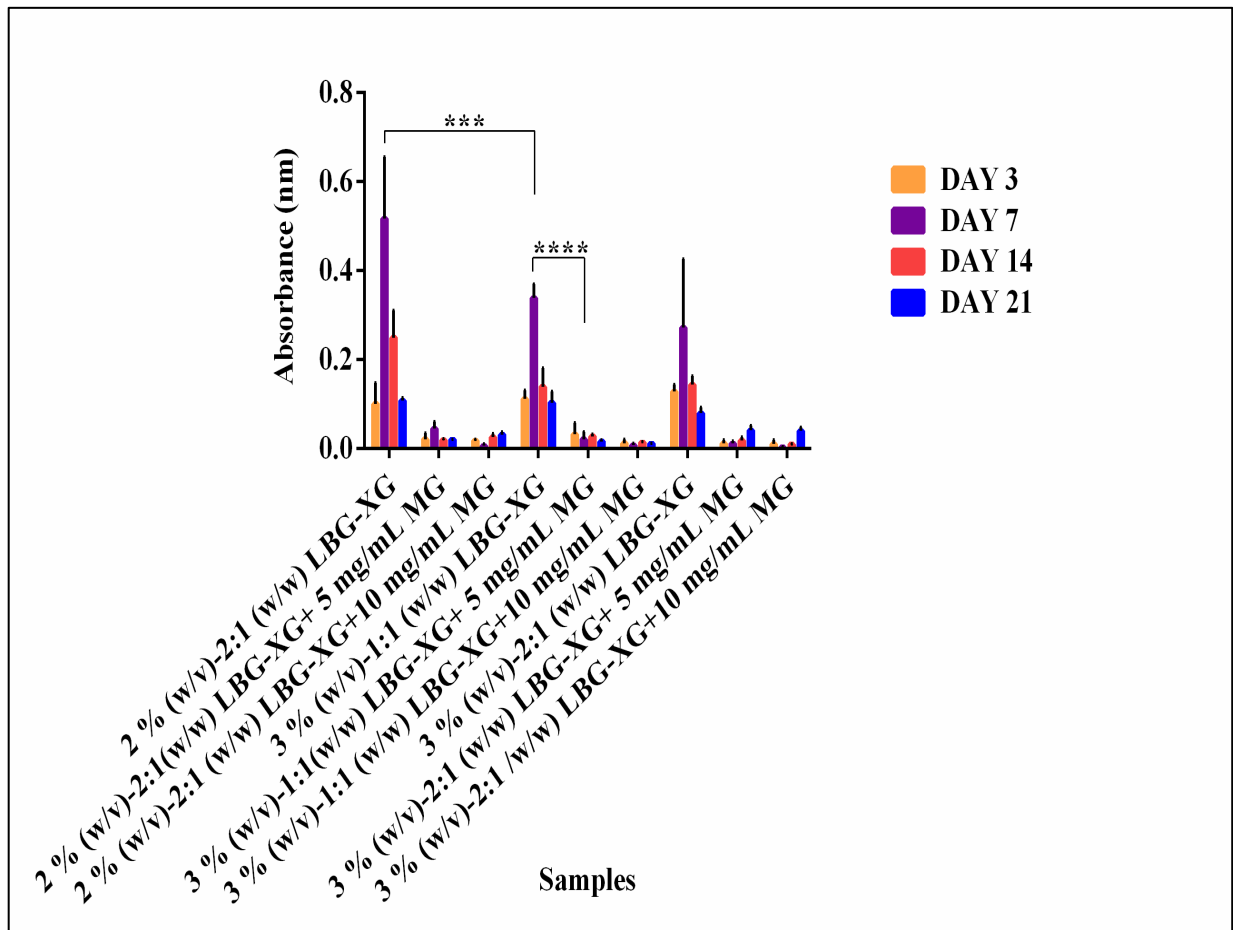


Figure 5.26. MTS cell viability assay performed for RBM-MSC-laden cryogels throughout 21 days of incubation (*** $P \leq 0.001$, **** $P \leq 0.0001$)

5.2.3.2. *Live/Dead® Cell Viability Assay*

Live/Dead® Cell Viability Assay was performed in order to visualize RBM-MSCs inside the cryogels. Calcein-AM and ethidium homodimer-1 are fluorescent dyes that are used to stain live and dead cells. Green fluorescent and red fluorescent dyes indicated live and dead cells, respectively. The cells were observed under fluorescent microscope on days 3, 7, 14 and 21. Cell-free cryogels were used as control groups to determine whether blank cryogels luminescent or not, so that the live cells could be easily distinguished from cryogels. Figures 5.27 and 5.28 show fluorescently labelled cells inside the scaffolds. The cells were attached to the cryogels and labelled with Calcein-AM on day 3. From day 3 to day 7, the number of fluorescently labelled cells increased in all cryogels (Figure 5.27). In Figure 5.28, it was observed that more cells become aggregates from day 14 to day 21, especially in samples 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG. From day 7 to day 14, the number of the cells observed inside 2 percent LBG-XG cryogel set decreased, which was similar with the results of MTS assay. On day 28, 3 percent LBG-XG containing cryogels had more cells inside than 2 percent LBG-XG cryogels. On the other hand, the number of cells increased from day 3 to day 28 in contrast with the MTS results. There were hardly any dead cells observed inside the cryogels, so that color red could not be seen in images. The live/dead assay reveals that all cryogels provided a suitable environment for cells to grow and proliferate. When the results of MTS and live/dead assay compared, differences were observed in cell proliferation profile. In MTS, absorbance values of MG containing cryogels were less than MG-free ones. However, in live/dead assay it was seen that the cells were able to proliferate in MG containing cryogels as well as MG-free ones. In addition, the number of fluorescently labelled cells inside 2 percent (w/v) - 2:1 (w/w) LBG-XG, 3 percent (w/v) - 1:1 (w/w) LBG-XG, 3 percent (w/v) - 2:1 (w/w) LBG-XG cryogels increased from day 3 to 21, unlike the results of MTS. The reason of these differences in two assays might related with the nature of cryogels. They might keep some of the formazan solution inside, and therefore the samples might give less absorbance at 490 nm. Live/dead assay proved that cryogels have favorable environment for RBM-MSC attachment, growth, and proliferation. The highest cell viability on day 21 was observed in 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL cryogels.

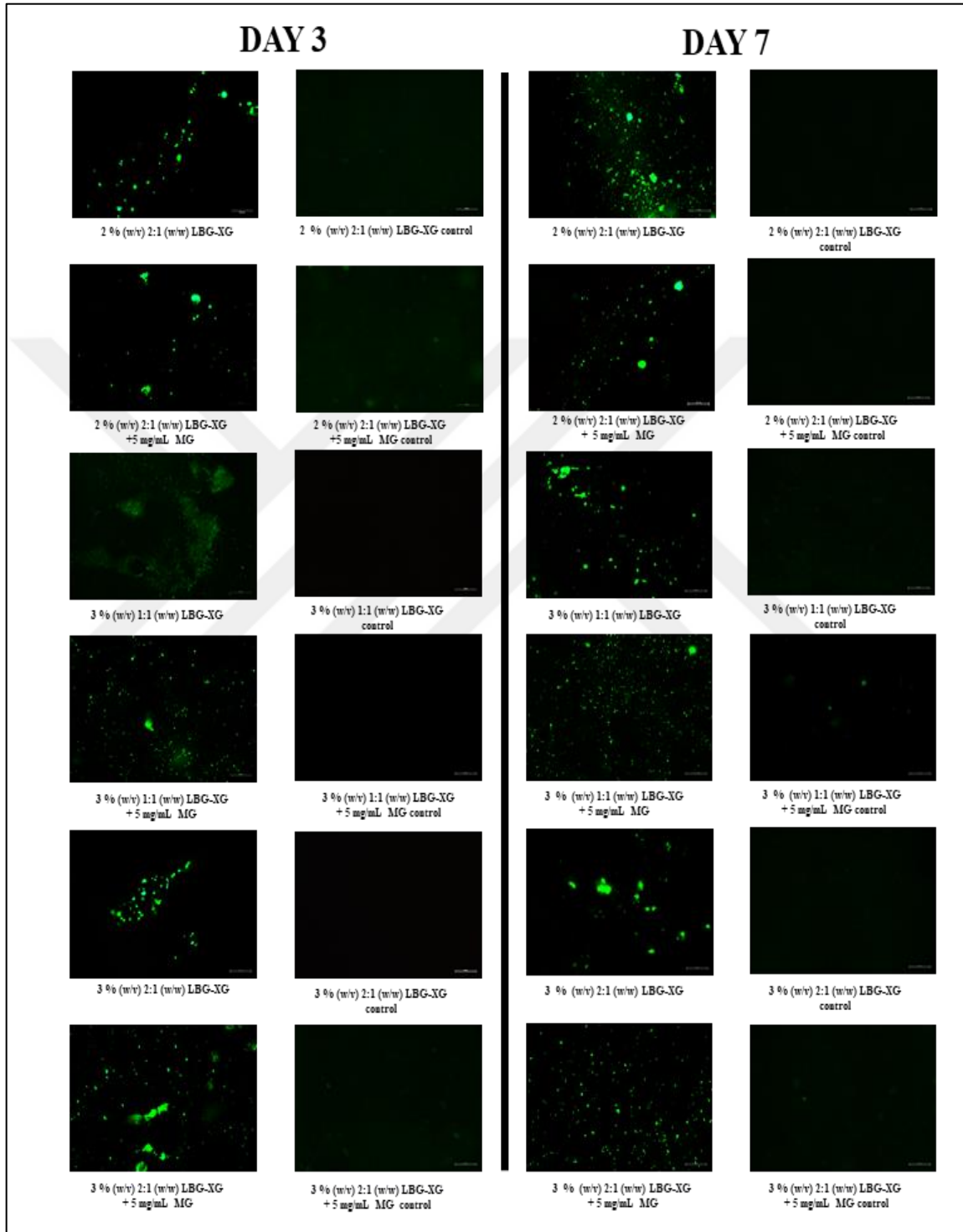


Figure 5.27. Calcein-AM (green) and Ethidium homodimer-1 (red) staining of RBM-MSCs inside the cryogels on days 3 and 7 (5 X magnification- scale bars:200 μ M)

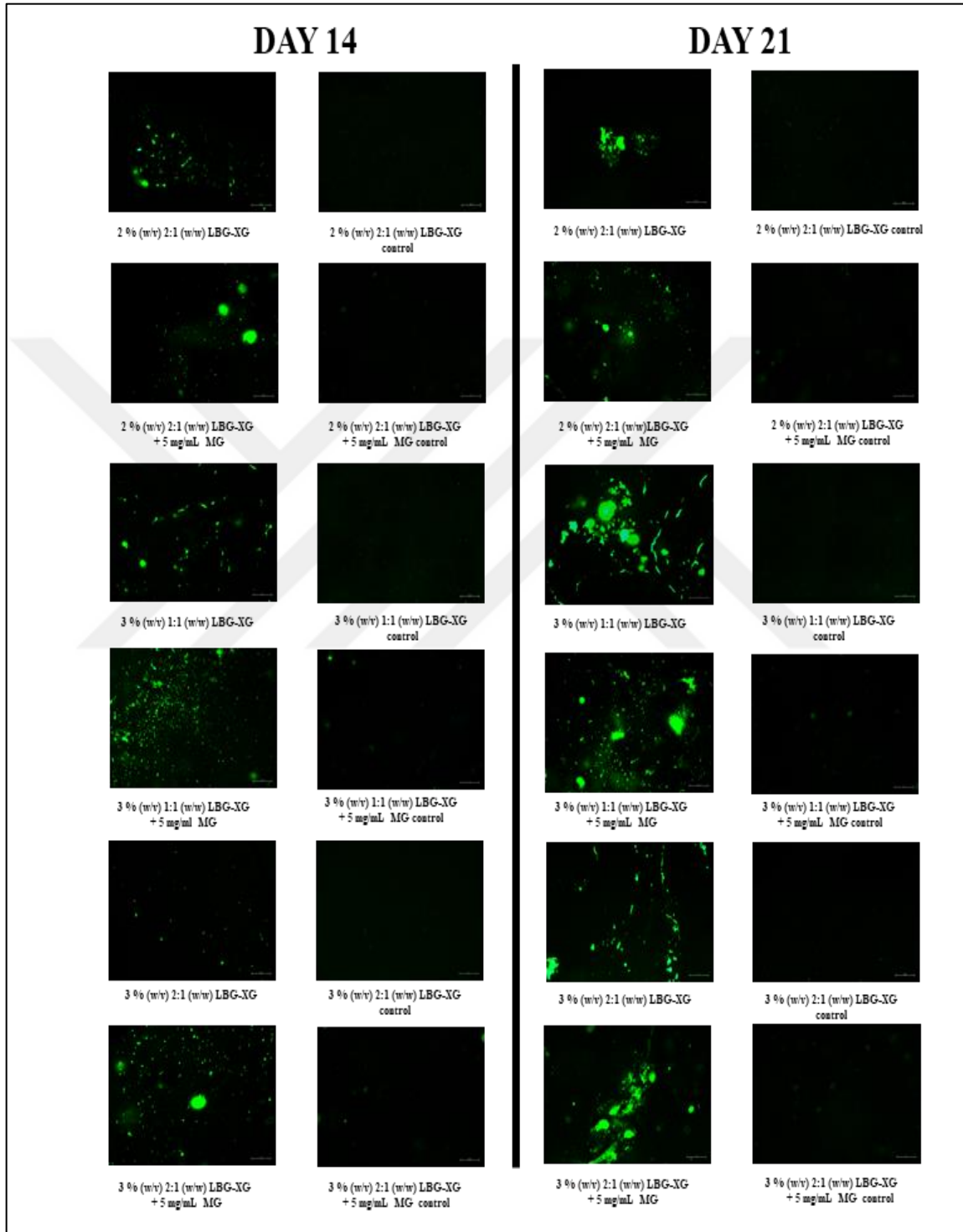


Figure 5.28. Calcein-AM (green) and Ethidium homodimer-1 (red) staining of RBM-MSCs inside the cryogels on days 14 and 21 (5 X magnification scale bars:200 μ m)

5.2.4. Chondrogenic Differentiation Capacity of RBM-MSCs in Cryogels

5.2.4.1. Alcian Blue Staining

Alcian Blue staining was performed to determine chondrogenic differentiation of RBM-MSCs seeded into cryogels. The images of cell-laden cryogels were taken under stereomicroscope. Cell-seeded and cell-free cryogels were incubated in KGN containing medium (KGN) and chondrogenic medium (CM) for 28 days. According to the characterization studies and cell viability assays, 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels were selected for further *in vitro* and *in vivo* studies.

The Alcian Blue staining results of cell-seeded and cell-free cryogels were compared in Figure 5.29. All cryogels stained pinkish red color, which might related with the polysaccharide nature of the cryogels. However, the cell-laden cryogels had blue-colored regions that were indicators of acid mucins, proteoglycans and hyaluronic acid. When cell-laden cryogels incubated in CM, cartilage specific matrix molecule production was increased from day 7 to 28. Blue colored regions in 3 percent (w/v) - 1:1 (w/w) LBG-XG were more than the ones in 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels on 28th day, meaning that 3 percent (w/v) - 1:1 (w/w) LBG-XG cryogels incubated in CM provided more suitable environment for cells to differentiate into chondrocytes. On the other hand, cell-laden cryogels incubated in KGN containing medium produced more cartilage specific matrix molecules than the ones incubated in CM on day 28. There weren't any significant difference in the matrix production of cells seeded in 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL cryogels incubated in KGN containing medium.

All cryogels supported the chondrogenic differentiation of RBM-MSCs with respect to the results of Alcian Blue staining. However, the cells incubated in KGN containing medium had more blue regions compared with the cells incubated in CM.

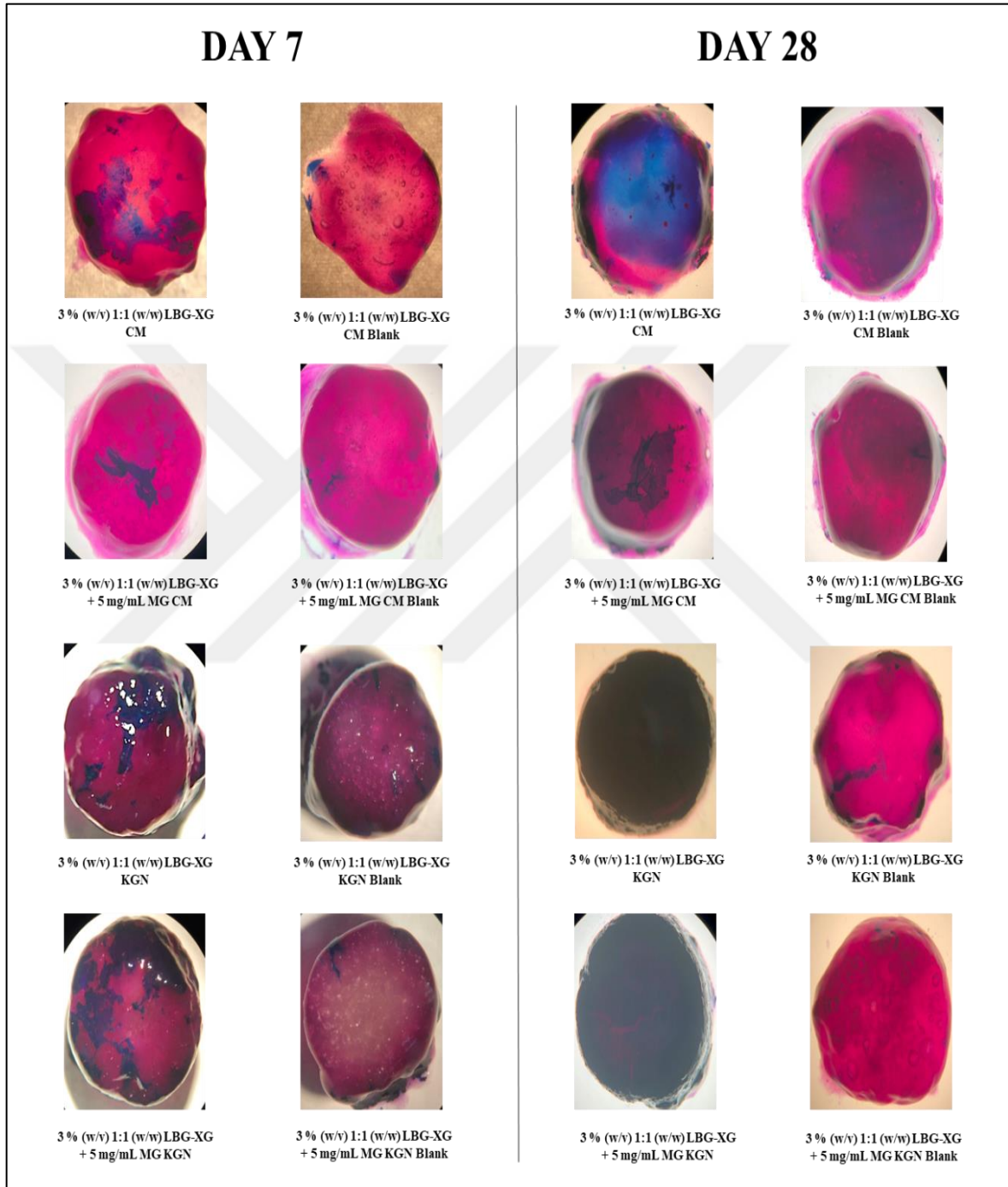


Figure 5.29. Alcian Blue stainings of cell-laden and cell free (Blank) 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels incubated in KGN containing medium (KGN) and chondrogenic medium (CM) on days 7 and 28.

(Images were taken under stereomicroscope)

5.2.4.2. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative polymerase chain reaction analyses were performed to determine the expression of SOX9, COL2A1, ACAN, RUNX1, and COL1A1 genes upon induction of RBMSCs inside cryogels with CM and KGN containing growth medium (KGN). The SOX9 is a key transcription factor that regulates early chondrogenesis of MSCs. Besides its role in chondrogenic differentiation, SOX9 is also needed to perpetuate proliferation and viability of growth plate chondrocytes and delays the prehypertrophy [264]. Upon upregulation, SOX9 activates the expression of other cartilage specific genes COL2A1 and ACAN [265]. In the proliferative phase of chondrocytes, COL2A1 and ACAN are highly expressed [197]. Type II collagen protein production can promote chondrogenesis and maintain the expression of chondrocytic phenotype [266]. Aggrecan is the second major molecule produced during chondrogenesis. It can improve flexibility, viscoelasticity and load bearing capacity of the musculoskeletal tissues by retaining water inside [14,267]. Apart from these, RUNX1 associates with SOX9 to enhance cartilage matrix production and to suppress hypertrophic differentiation of articular chondrocytes. Moreover, RUNX1 is expressed by chondrogenic progenitor cells and participates in early chondrogenic differentiation [268]. The expression of RUNX1 is also associated with the KGN small molecule. When RUNX1 gene was knocked down in human bone marrow MSCs, chondrogenic differentiation upon KGN induction was obstructed [201]. The stimulatory effect of KGN on chondrogenic differentiation of MSCs or progenitor cells has been proven in many studies so far [242, 253, 268-273]. The expression of a fibroblastic and osteocytic marker COL1A1 gene was also determined among chondrogenic marker genes SOX9, COL2A1, ACAN and RUNX1.

The gene expression values were represented as fold change with respect to untreated RBMSCs seeded inside the cryogels. The fold change values calculated via $2^{-\Delta\Delta C_q}$ method. Statistical significance was determined with multiple comparison Tukey's test following two-way Anova. Figure 5.30 shows the expression of early marker genes SOX9 and RUNX1. The fold change of SOX9 and RUNX1 genes were compared in cell-laden cryogels having 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG concentrations. The SOX9 expressions of all samples on day 3 were higher than the ones on day 7 (Figure 5.30 a).

The higher expression of SOX9 on early phases of induction can be explained by being an early marker and the main regulatory gene for chondrogenesis. A slight increase was observed in SOX9 fold change expression from day 7 to day 14 in 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN) samples. Furthermore, there was a 25-fold decrease observed in SOX9 expression of the same sample from day 3 to day 28. The 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG (KGN) samples showed similar SOX9 expression profile with 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN) samples. However, MG containing samples incubated in KGN expressed significantly higher SOX9 (~71 fold) than the MG-free ones on day 3. The expression of SOX9 significantly dropped from day 3 to day 28 in samples 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG (KGN). The RBMSCs in samples having MG incubated in KGN expressed ~84 and ~25 fold more SOX9 than the samples incubated in CM on days 3 and 18, respectively. Moreover, the 3 percent (w/v) - 1:1 (w/w) LBG-XG (CM) samples had the highest SOX9 expression among all samples on day 28.

The RUNX1 expression in MG containing samples decreased from day 3 to 28 (Figure 5.30 b). Cells in 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG (KGN) expressed significantly higher RUNX1 on day 3 than the other samples. The fold change expression of RUNX1 in samples in 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN) stayed nearly constant during first 18 days and then dropped below 1 on day 28. When the effects of KGN and CM on RUNX1 expression compared, it was observed that KGN treated samples expressed more RUNX1 than CM treated samples at early phases of chondrogenic induction. On the other hand, MG containing samples expressed higher RUNX1 on days 3 and 7 than the MG-free samples.

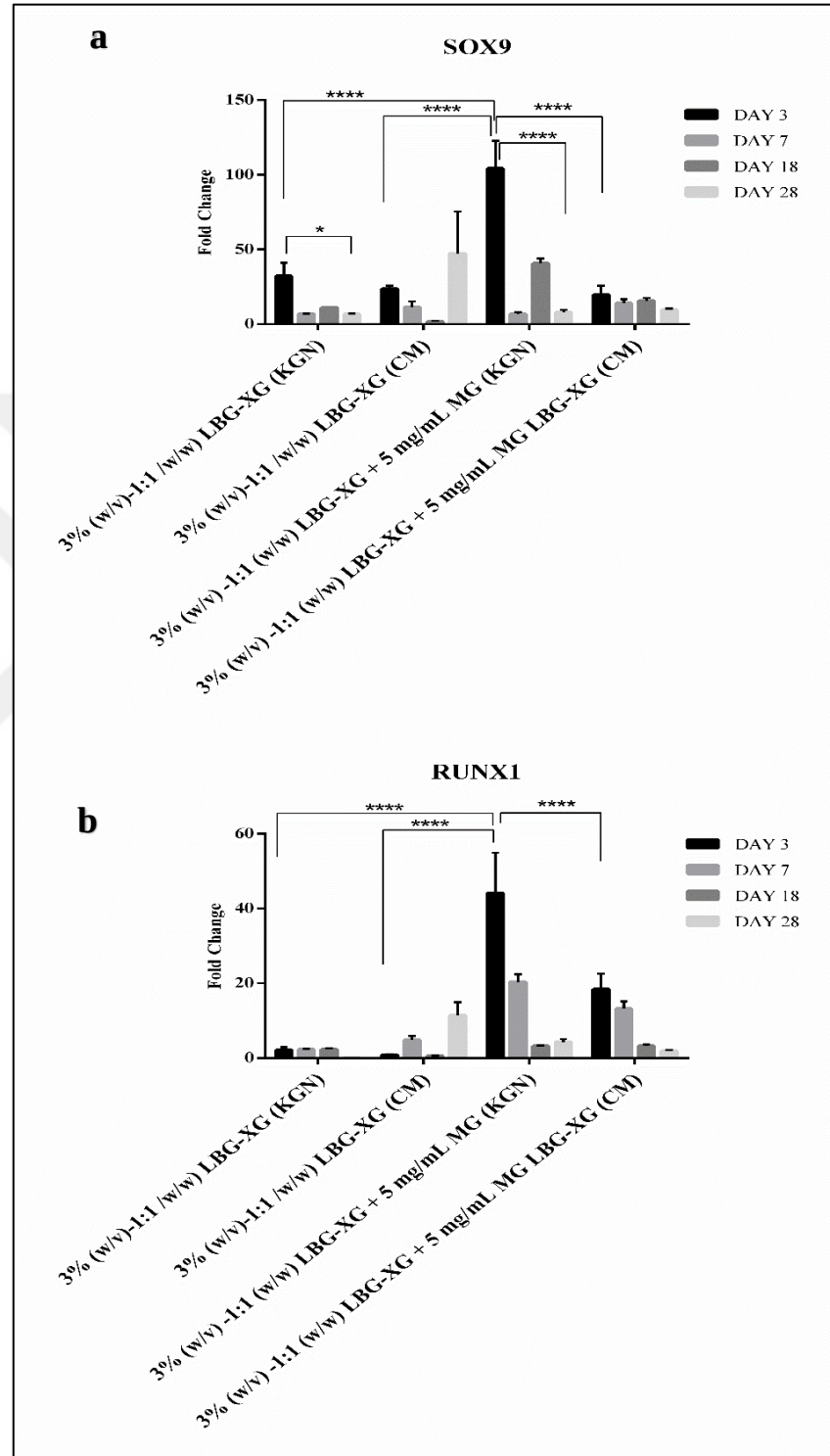


Figure 5.30. Expressions of (a) SOX9 and (b) RUNX1 genes represented as fold change ($2^{-\Delta\Delta C_q}$) with respect to untreated cell-laden cryogels (**** $P \leq 0.0001$)

The fold change expression values of COL2A1 and ACAN genes were demonstrated in Figure 5.31. Unlike SOX9 and RUNX1, COL2A1 and ACAN were generally expressed more at later phases of induction. The COL2A1 expression was increased from day 3 to 28 in all samples except 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN). The expression of COL2A1 in samples 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN) increased until 18 days, and then decreased approximately 48-fold from day 18 to day 28 (Figure 5.31 a). When the expression values within first 18 days were examined, it was realized that COL2A1 expressed in higher amounts in KGN treated samples than the samples incubated in CM. This might indicate that KGN triggered COL2A1 expression earlier than CM. The expression of COL2A1 in 3 percent (w/v) - 1:1 (w/w) LBG-XG (KGN) was significantly higher than the one in 3 percent (w/v) - 1:1 (w/w) LBG-XG (CM) on day 18. In contrast, 3 percent (w/v) - 1:1 (w/w) LBG-XG samples treated with CM expressed higher COL2A1 than the KGN treated ones on day 28. Similarly, the MG containing samples expressed significantly higher amount of COL2A1 when treated with KGN on day 18. On the other hand, MG containing samples incubated in CM expressed higher COL2A1 than the ones incubated in KGN on day 28.

Figure 5.31 b represents the fold differences of ACAN expression within the samples. The expression profiles of ACAN resemble each other in 3 percent (w/v) - 1:1 (w/w) LBG-XG samples. In a similar manner to MG-free samples, MG containing samples' expression profiles were also alike. The cells were able to express more ACAN in MG containing cryogels than the MG-free samples. There was no significant difference among 3 percent (w/v) - 1:1 (w/w) LBG-XG samples in terms of ACAN expression. However, the cells in MG containing cryogels expressed more ACAN than the cells in MG-free cryogels. Additionally, 3 percent (w/v) - 1:1 (w/w) LBG-XG +5 mg/mL MG (CM) samples expressed higher ACAN than 3 percent (w/v) - 1:1 (w/w) LBG-XG +5 mg/mL MG (KGN) samples on day 18. According to the results in Figures 5.30 and 5.31, the RBMSCs seeded in LBG-XG and LBG-XG-MG cryogels were able to express chondrogenic marker genes both in KGN containing medium CM. The SOX9 and RUNX1 genes expressed earlier and COL2A1 and ACAN genes expressed later phases of chondrogenesis as it was expected, especially in MG containing samples.

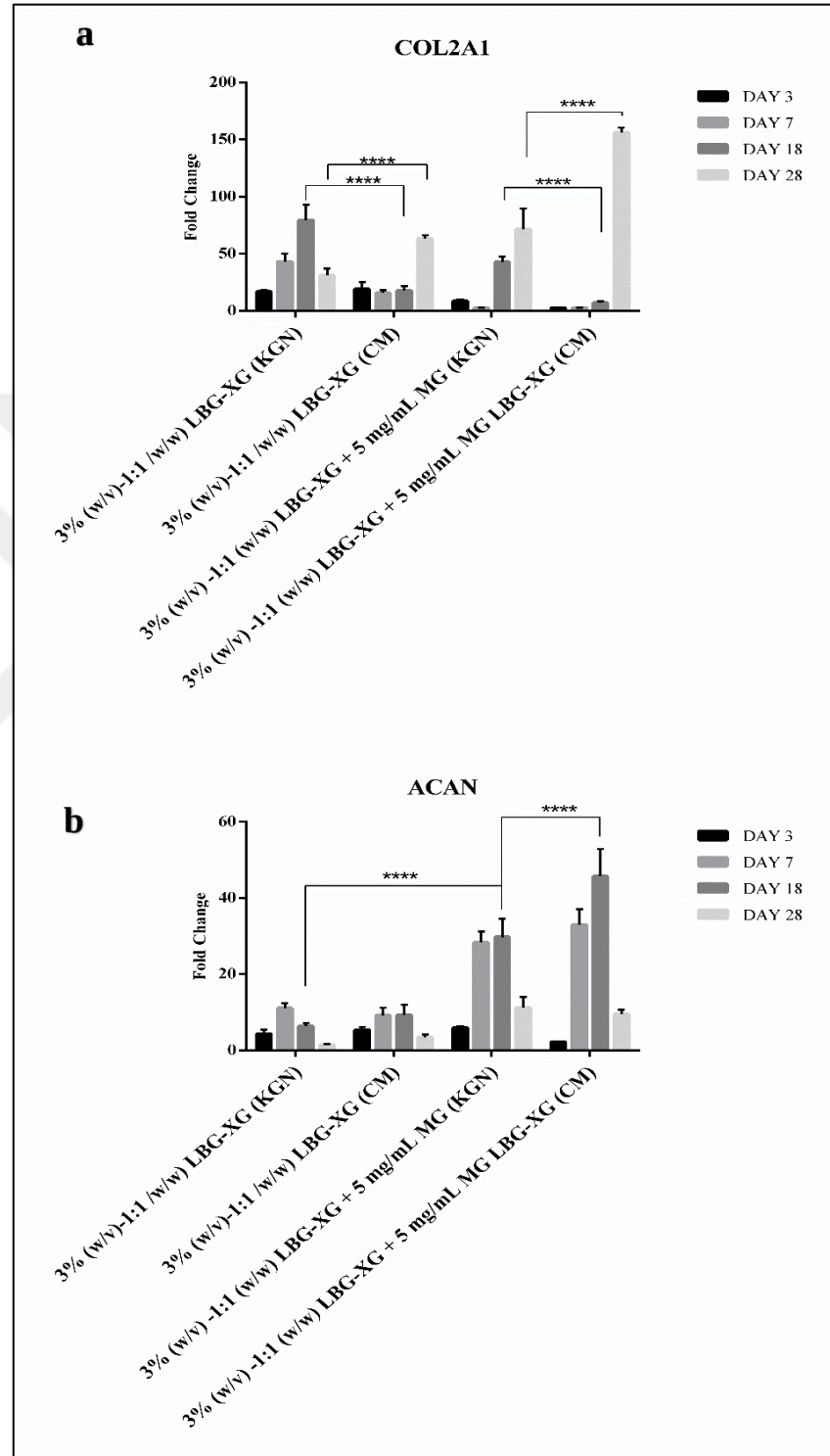


Figure 5.31. Expressions of (a) COL2A1 and (b) ACAN genes represented as fold change ($2^{-\Delta\Delta C_q}$) with respect to untreated cell-laden cryogels (**** $P \leq 0.0001$)

The expression of COL1A1 gene was also determined in order to examine osteocytic or fibroblastic differentiation of cells throughout 28 days (Figure 5.32 a). The COL1A1 expression in 3 percent (w/v) - 1:1 (w/w) LBG-XG samples decreased from day 3 to day 28. There was a significant difference in COL1A1 expression between KGN and CM treated 3 percent (w/v) - 1:1 (w/w) samples on day 28. However, since the fold change values were below 1, it can be deduced that the cells in treated cryogels expressed less COL1A1 than the ones in non-treated samples. On the other hand, in MG containing samples, there were approximately 2-fold and 7.5-fold decrease in COL1A1 expression when cells treated with KGN and CM respectively. The cells expressed ~10.5-fold and ~3-fold more COL1A1 on day 28 than non-treated samples. Although it seems like some cells differentiated into fibroblast like or osteocytic cells in MG containing cryogels on day 28, they expressed 7-fold and 56-fold more COL2A1 than COL1A1 in KGN containing media and CM, respectively according to Figure 5.32 b. The MG-free samples expressed higher COL2A1 than the MG containing samples with respect to the COL2A1/COL1A1 ratio.

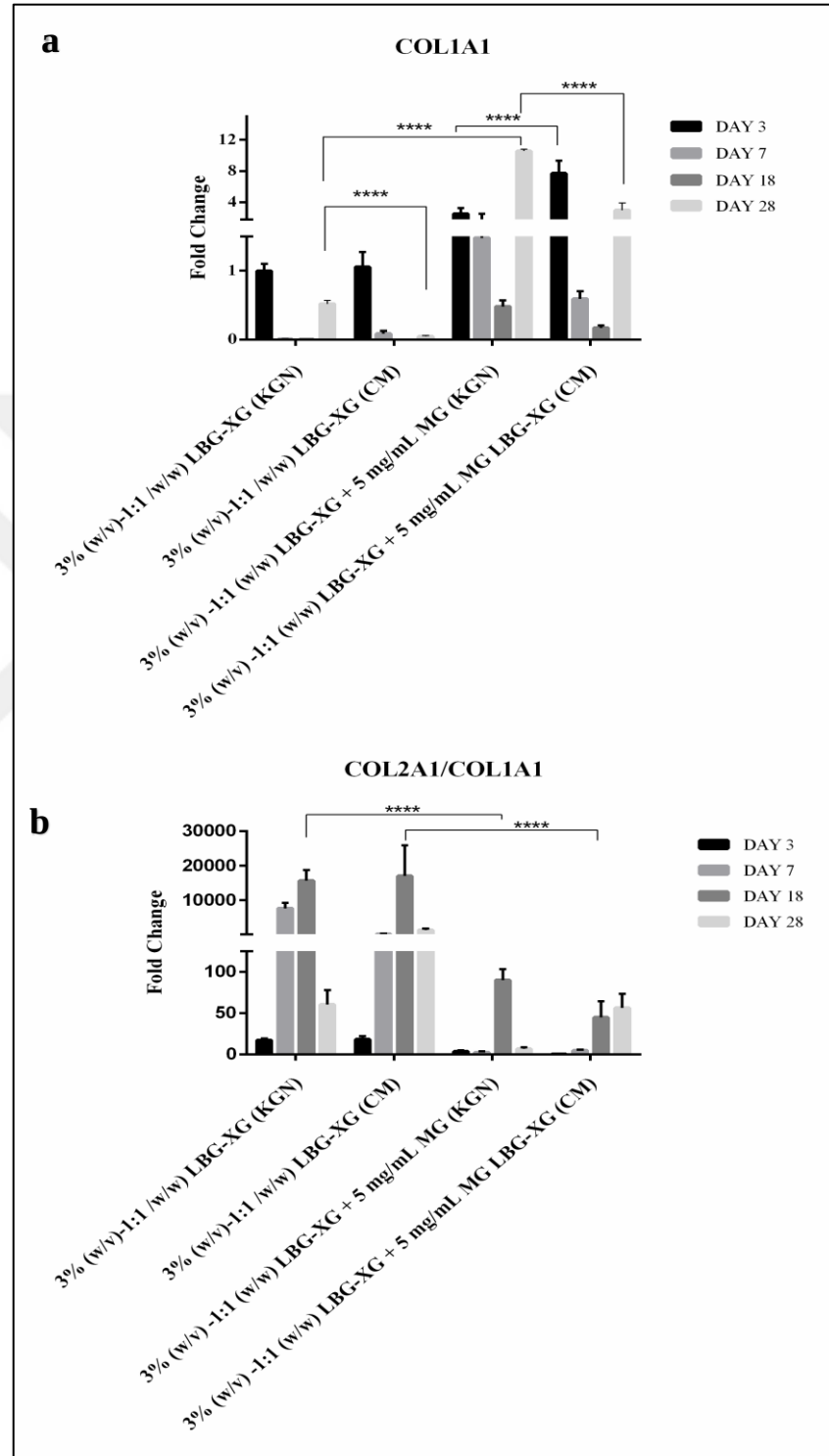


Figure 5.32. Expressions of (a) COL1A1 gene and (b) COL2A1/COL1A1 ratio represented as fold change ($2^{-\Delta\Delta C_q}$) with respect to untreated cell-laden cryogels (**** $P \leq 0.0001$)

5.2.4.3. Immunocytochemistry Analysis

The effect of KGN on chondrogenic differentiation was also examined by immunocytochemical analysis. The RBM-MSC seeded cryogels having concentrations of 3% (w/v) - 1:1 (w/w) LBG-XG and 3% (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG were stained with anti- type II collagen and anti-aggrecan antibodies on 7th and 28th days of KGN induction. The nuclei of the cells were stained with DAPI (blue) in order to discriminate cells from the other elements inside the cryogels. In the proliferative state, chondrocytes highly produce type II collagen and aggrecan [197], therefore it is important to observe the production of these cartilage specific matrix molecules by RBM-MSCs treated with KGN, in order to prove chondrogenic differentiation. According to Figure 5.33, the RBM-MSCs inside both cryogels were able to produce ECM molecule type II collagen in small amounts on day 7 (Figure 33 a, c), as it was expected. However, cells did not start to produce aggrecan on 7th day of KGN induction (Figure 5.33 c). Figures 5.33 b and d reveal the increasing fluorescence intensity of type II collagen and aggrecan in both samples on day 28. This situation indicates the increase in type II collagen – aggrecan production from day 7 to day 28. When Figures 5.33 b and d were compared, it was observed that the cells inside the 3 % (w/v) - 1:1 (w/w) LBG-XG cryogels came together and formed clusters. On the other hand, the cells scattered inside the 3% (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels (Figure 5.33 d).

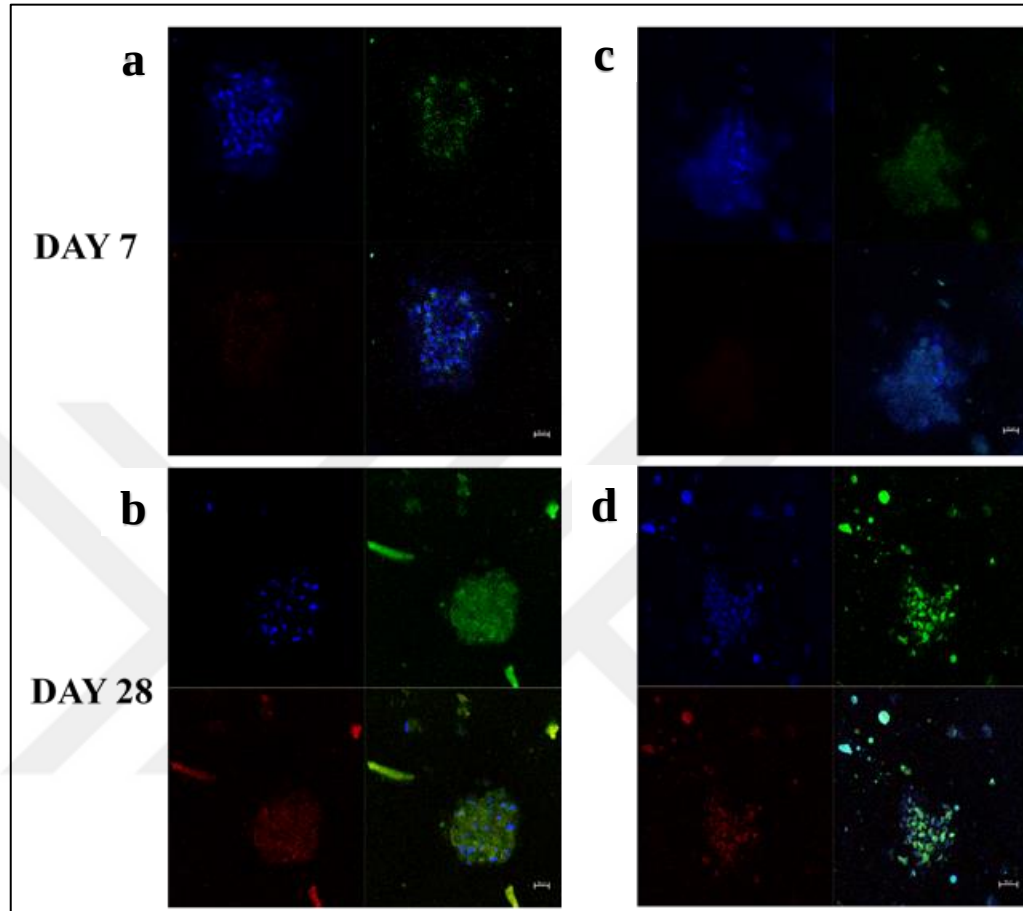


Figure 5.33. The type II collagen-aggrecan immunostaining of RBM-MSC seeded cryogels having concentrations of (a, b) 3% (w/v) - 1:1 (w/w) LBG-XG, (c, d) 3% (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG on (a, c) 7th and (b, d) 28th days of KGN induction. (a,b,c : 20X magnification - scale bars: 20 μ M, d:10X magnification – scale bar: 50 μ M), (Blue:nuclei, green: type II collagen, red: aggrecan)

5.3. *IN VIVO* ANALYSIS OF CRYOGELS

Cartilage development begins with a condensation step; in which mesenchymal chondroprogenitor cells come together and then differentiate into chondrocytes. It continues with cartilage specific ECM production mostly comprising type II collagen and aggrecan. Afterwards, chondrocytes surrounded by their ECM and exhibit rounded morphology [11]. In articular cartilage, chondrocytes, that are able to proliferate, undergo a process named interstitial growth. After cell division, chondrocytes stay close to each other by enlarging their surrounding pericellular matrix. The structure consisting of such group of cells (isogenous cell groups) encircled by a pericellular matrix is called chondrone [21, 274]. The pericellular matrix has significant roles in transducing biomechanical and biochemical signals to the chondrocytes. It also protects cells from mechanical stress thus, preventing articular cartilage from degradation [275, 276]. For *in vivo* studies, 16 weeks old male Sprague Dawley Rats were used. Cell-laden and cell-free cryogels were implanted subcutaneously to the back of the rats, and after 4 and 8 weeks, the implants were taken out and sectioned for histological evaluation. The KGN-loaded cryogels were compared with KGN-free ones in terms of chondrogenic differentiation. Cell-free cryogels were used as a control group. Figures 5.34 and 5.35 show cryogel sections with or without MG stained with Alcian Blue dye 4 weeks and 8 weeks after implantation, respectively. The black arrows denote the fibrous tissue surrounding cryogels without cells. There was no RBM-MSCs like cells inside the cryogels; however, some cells migrated into cryogels from the surrounding tissue. When the cells inside the KGN-loaded and KGN-free cryogels investigated, some differences were observed regarding their morphology. The KGN-treated cells became more rounded and differentiated into chondrocyte-like cells (red arrows). The cells inside KGN-free cryogels remained more like MSCs, displaying polygonal shapes [277]. However, a few cells with rounded morphology were also observed in KGN-free cryogels. Among all samples, the 3 percent (w/v) - 1:1 (w/w) LBG-XG KGN loaded cryogels had the cells with a morphological resemblance to the hyaline cartilage tissue. Furthermore, the cryogels were all stained blue (indicated as green arrows), since they are polysaccharide based.

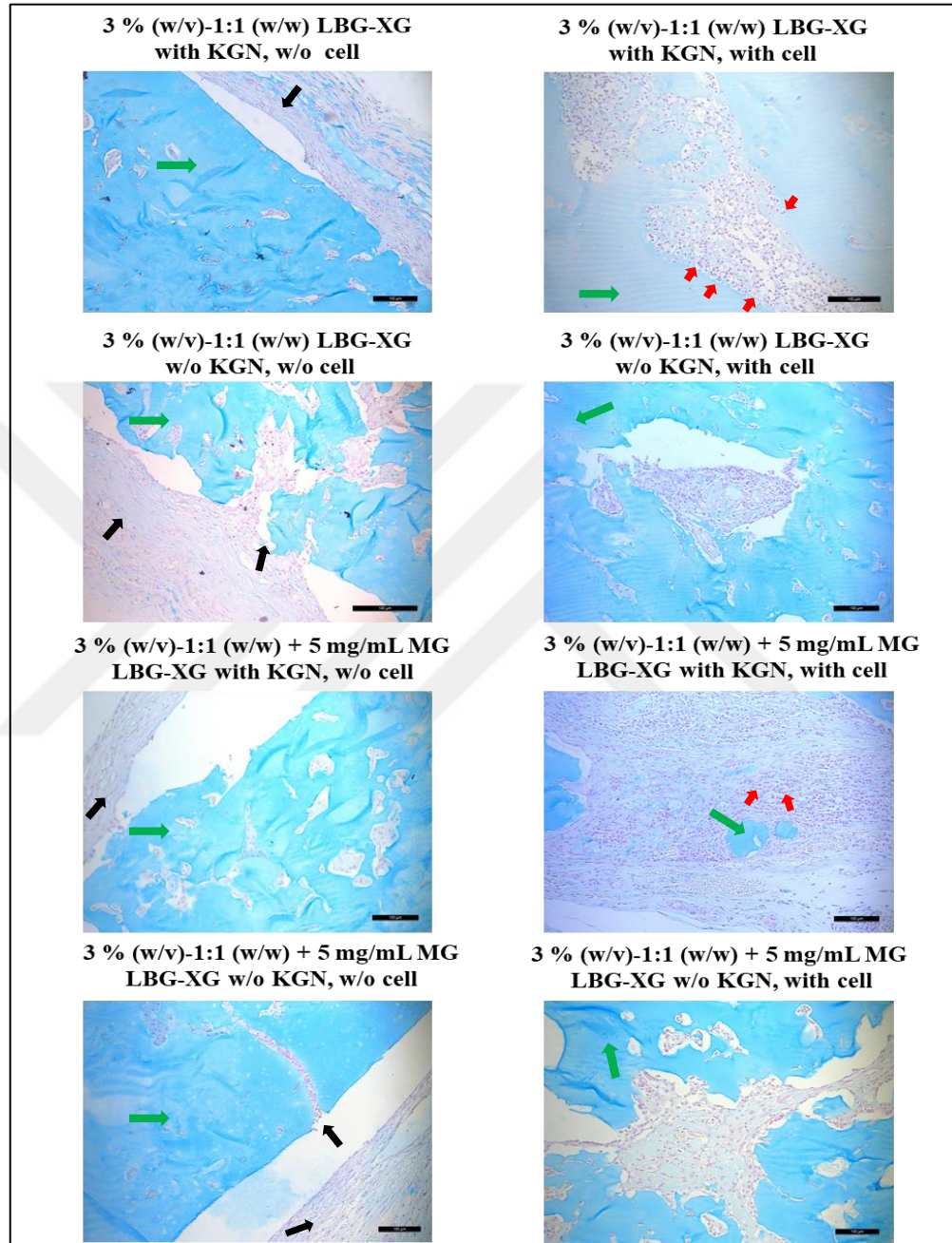


Figure 5.34. Histological images of the Alcian Blue staining of KGN-loaded and KGN-free 3 percent (w/v)-1:1 (w/w) LBG-XG and 3 percent (w/v)-1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels with or without cells 4 weeks after implantation. (black arrows: fibrous tissue, red arrows: chondrocyte-like cells, green arrows: cryogels) (20 X magnification- scale bars:50 μ M)

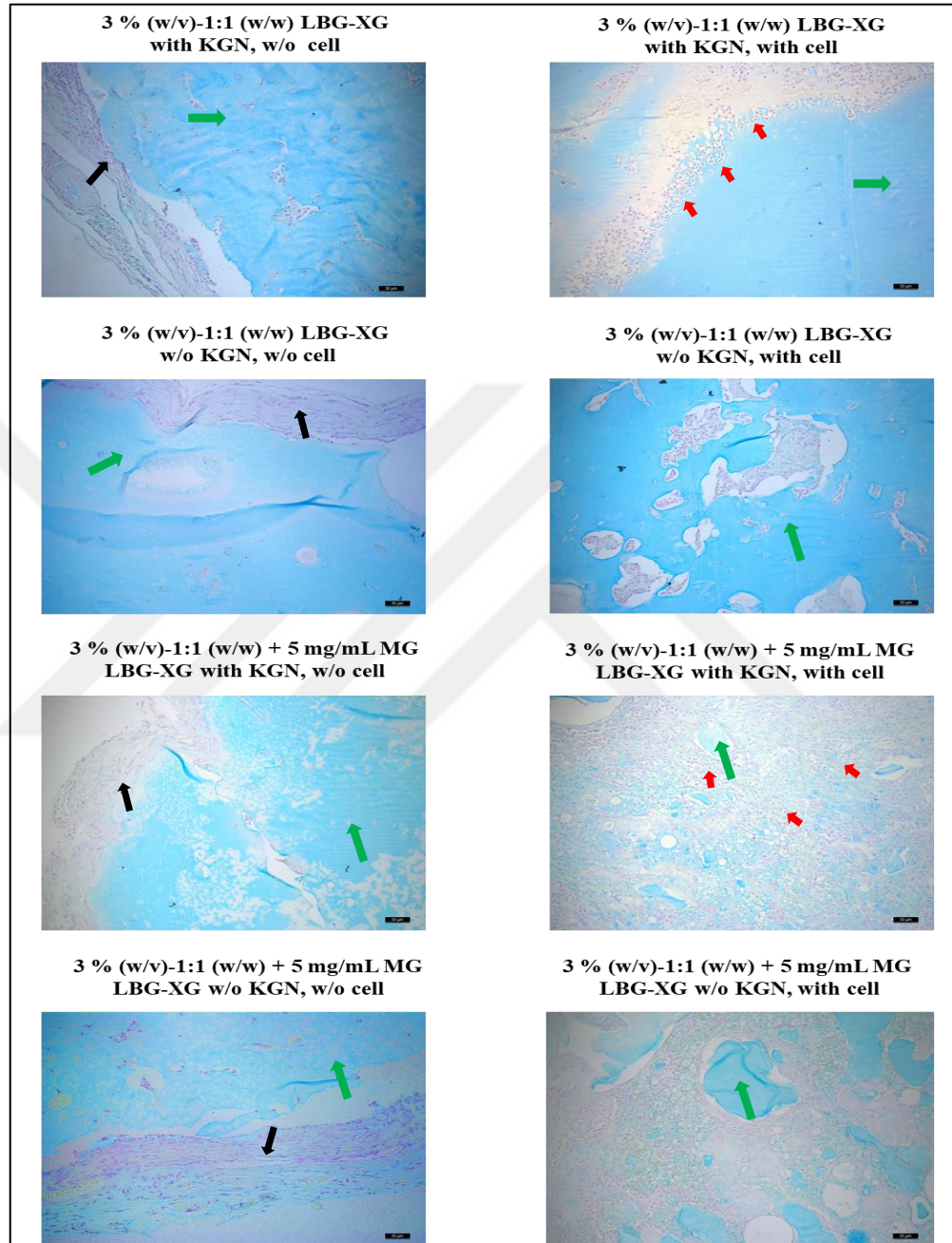


Figure 5.35. Histological images of the Alcian Blue staining of KGN-loaded and KGN-free 3 percent (w/v)-1:1 (w/w) LBG-XG cryogels and 3 percent (w/v)-1:1 (w/w) LBG-XG + 5 mg/mL MG with or without cells 8 weeks after implantation. (black arrows: fibrous tissue, red arrows: chondrocyte-like cells, green arrows: cryogels) (20 X magnification-scale bars:50 μ M)

Figures 5.36 and 5.37 demonstrate more detailed images of Alcian Blue stainings of cell-laden KGN-loaded and KGN-free cryogels, 4 and 8 weeks after implantation. The effect of KGN on chondrogenic differentiation can be easily observed by the existence of chondrocyte-like cells (isogenous cell groups) and chondron-like structure formation (red arrows) in KGN-loaded 3 percent (w/w)-1:1 (w/w) LBG-XG cryogels (Figure 5.36). The MG containing, KGN-loaded cryogels also had chondrocyte like cells after 4 and 8 weeks of implantation (Figure 5.37). However, there were more chondrocyte-like cells in 3% (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels after 8 weeks of implantation compared to 4 weeks. Moreover, the cells inside 3% (w/v) - 1:1 (w/w) LBG-XG formed clusters resembling the native hyaline cartilage tissue. The 3% (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG cryogels did not have such structures, although they had differentiated chondrocyte-like cells after 4 and 8 weeks of implantation.

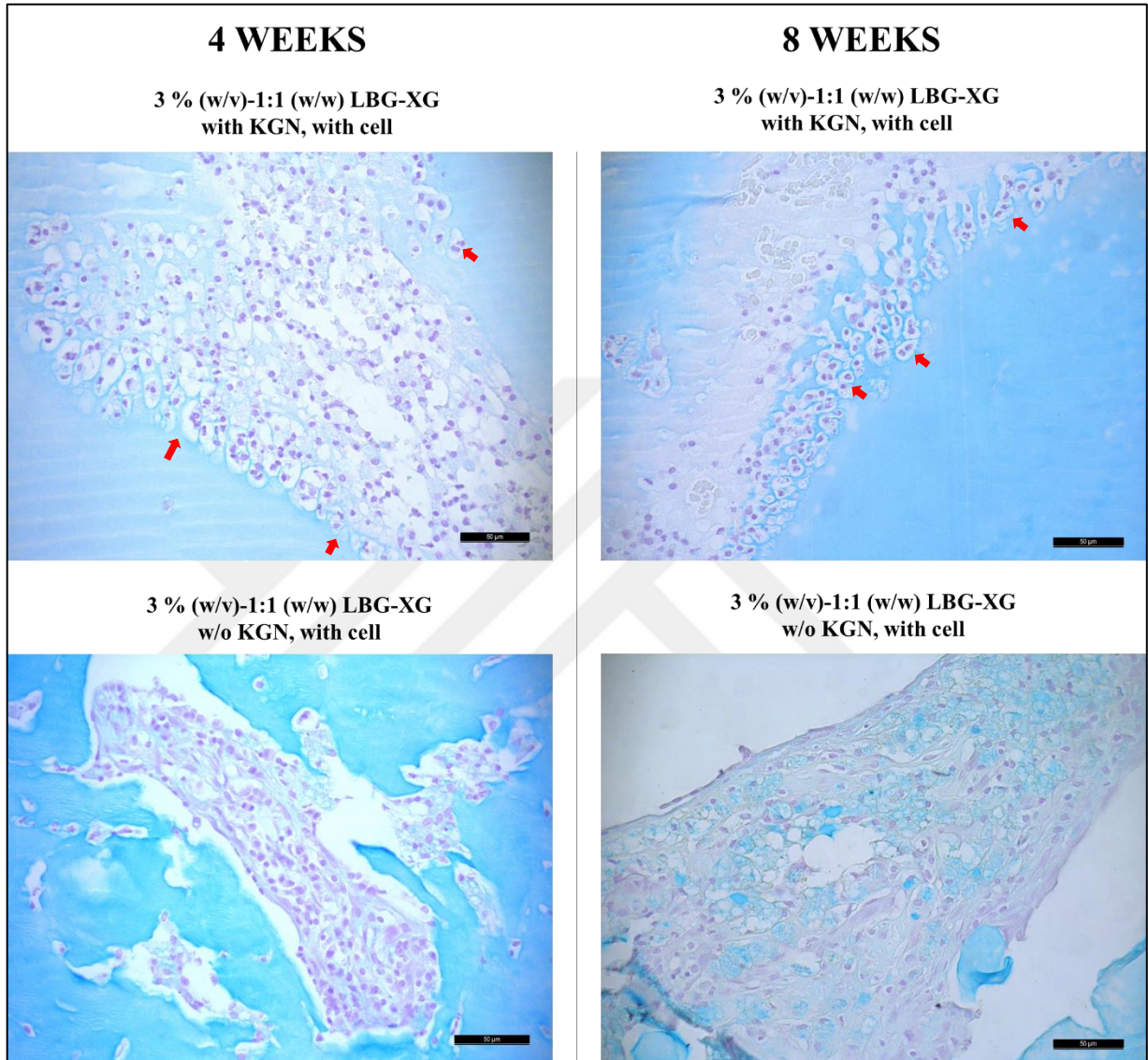


Figure 5.36. Histological images of the Alcian Blue staining of KGN-loaded and KGN-free 3 percent (w/w)-1:1 (w/w) LBG-XG cryogels with cells 4 and 8 weeks after implantation. (red arrows: chondrocyte-like cells and chondron-like structures) (40 X magnification- scale bars:50 μ M)

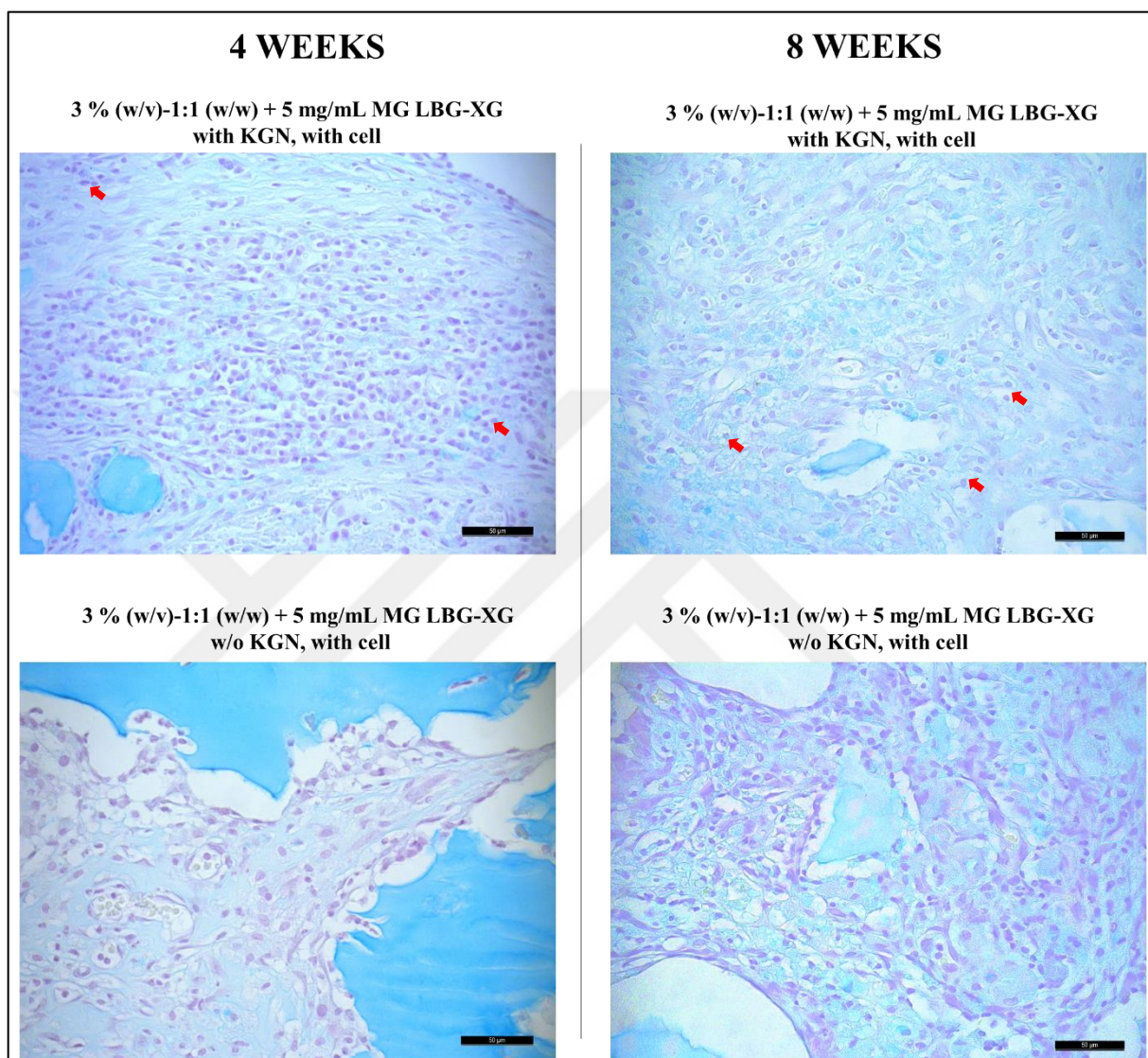


Figure 5.37. Histological images of the Alcian Blue staining of KGN-loaded and KGN-free 3 percent (w/w)-1:1 (w/w) LBG-XG 5 mg/mL MG cryogels with cells 4 and 8 weeks after implantation. (red arrows: chondrocyte-like cells chondron-like structures) (40 X magnification- scale bars:50 μ M)

6. CONCLUSION

Cartilage tissue engineering is a research field that is applied when conventional treatment methods are insufficient to treat damaged cartilage tissues. The aim of cartilage tissue engineering is to design biological substitutes which can replace damaged tissues due to disease, trauma, or congenital disorders. The ideal cartilage tissue engineering construct should mechanically and structurally resemble the native cartilage tissue and promote chondrogenic differentiation [278]. The purpose of this study was to create cartilage tissue engineering constructs by combining natural biomaterials LBG, XG and MG varying concentrations in the form of cryogels. First, SEM analysis was performed to examine the physical appearance of cryogels. It demonstrated the interconnected pore structures of LBG-XG and LBG-XG-MG cryogels with pore sizes mostly in-between 0-400 μM . Cryogels have interconnected polymeric network with larger pores that allow nutrient and waste transportation, cellular infiltration and organization, and proliferation of cells [279]. Afterwards, LBG-XG and LBG-XG-MG cryogels were mechanically and chemically characterized. Mechanical analysis revealed that cryogels had similar mechanical properties with the native cartilage and other polysaccharide-based cryogels. The results of chemical characterizations were indicated the successful blend formation of LBG-XG and LBG-XG-MG cryogels. Biodegradability of the tissue engineering constructs is an important phenomenon since they should degrade in an appropriate amount of time for the replacement of the native tissue [280]. The LBG-XG and LBG-XG-MG cryogels were able to degrade without causing a dramatic decrease in the pH values of medium. In order to induce chondrogenic differentiation of RBM-MSCs seeded into cryogels, the KGN small molecule was loaded into them and release profile was determined. According to these results, LBG-XG and LBG-XG-MG cryogels can be used for sustained release of KGN, thus increase its effectiveness for chondrogenic differentiation. The cell-matrix interactions were examined through MTS cell viability and Live/Dead assays. The RBM-MSCs were able to attach, grow and proliferate inside cryogels. However, cryogels having concentrations of 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL MG had better mechanical and physical properties compared to other cryogels, therefore, further analysis

performed with these concentrations. Alcian Blue staining and immunocytochemical analysis showed cartilage-specific ECM, type II collagen and aggrecan production by RBM-MSCs inside cryogels. Furthermore, upregulation of chondrogenic marker genes; SOX9, RUNX1, COL2A1, ACAN, and downregulation of osteocytic marker gene COL1A1 were determined via qRT-PCR. Apart from these, 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL KGN-loaded and KGN-free cryogels were implanted subcutaneously with or without cells to the back of Sprague-Dawlet rats. Regarding Alcian Blue stainings of the implants taken out after 4 and 8 weeks of implantation, RBM-MSCs were able to differentiate into chondrocyte like cells inside the KGN-loaded cryogels.

In the light of these results, it was proven that 3 percent (w/v) - 1:1 (w/w) LBG-XG and 3 percent (w/v) - 1:1 (w/w) LBG-XG + 5 mg/mL cryogels are able to support and promote chondrogenic differentiation of RBM-MSCs upon induction with KGN small molecule both *in vitro* and *in vivo*. Moreover, these cryogels can be promising candidates for cartilage tissue engineering applications.

7. FUTURE PROSPECTS

The aim of this study was to investigate chondrogenic differentiation capacity of RBM-MSCs inside LBG-XG and LBG-XG-MG cryogels with the help of chondrogenic stimulator KGN. According to the results, the aim was accomplished both *in vitro* and *in vivo*. However, some additional trials can be done in order to reinforce physical and mechanical properties of cryogels. The freezing time, number of freeze-thaw cycles, and cooling rate can be changed to increase physical crosslinking, so that mechanical properties can also be improved. Along with physical crosslinking, chemical crosslinking can be done with different chemicals such as glutaraldehyde, but they may have some adverse effects on cells due to their toxicity. Apart from these, RBM-MSCs can be treated with higher concentrations of KGN to see whether chondrogenic differentiation is affected by increasing KGN concentration or not. The cell seeding density can also be increased in order to perform easy *in vitro* examinations. Furthermore, *in vivo* chondrogenic differentiation capacity of cell-seeded cryogels can be examined in different animal models and implantation areas.

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