Avrupa Bilim ve Teknoloji Dergisi
Sayı 41, S. 59-66, Kasım 2022

Araştırma Makalesi

www.ejosat.com ISSN:2148-2683

European Journal of Science and Technology
No. 41, pp. 59-66, November 2022

Research Article

İnsan Mezenkimal Kök Hücrelerinin Heparin Mimetik Peptit ile Modifiye Edilmiş Kendiliğinden Yapılandırılmış Peptit Hidrojel ile Arttırılmış Proliferasyonu

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(İlk Geliş Tarihi 24 Mart 2022 ve Kabul Tarihi 14 Eylül 2022)

(**DOI:** 10.31590/ejosat.1086807)

ATIF/REFERENCE: Sunal, G., Pulat, G., & Karaman, O. (2022). Enhanced Proliferation of Human Mesenchymal Stem Cells by Self-Assembled Peptide Hydrogel Modified with Heparin Mimetic Peptide. *Avrupa Bilim ve Teknoloji Dergisi*, (41), 59-66.

Öz

Doğal hücre dışı matris (HDM) bileşenlerinin rolünü taklit eden üç boyutlu (3B) mikro ortamların geliştirilmesi çok önemlidir. Kendiliğinden yapılanan peptit (KYP) hidrojeller, hücrelerin enkapsülasyonu, çoğalması, 3B kültürü, ve farklılaşması için ve ayrıca kıkırdak dokusundaki kusurları onarmak için yapı iskeleleri olarak kullanılmıştır. KYP'ler, biyomimetik yapı iskeleleri oluşturmak için kısa peptit epitopları ile biyofonksiyonelleştirilebilir. Burada, KLD (KLDLKLDLKLDL) KYP, KLD-HM (KLDLKLDLKLDL-EGDK) KYP hidrojeli üretmek için kısa bir biyoaktif motif olan EGDK ile işlevselleştirildi ve kendiliğinden yapılandırıldı. Mevcut çalışmada amaç, geliştirilen KLD-HM KYP hidrojellerinin insan mezenkimal kök hücrelerinin (iMKH'ler) canlılığı ve proliferasyonu üzerindeki etkisinin araştırılmasıdır. KYP'lerin stabilitesini sağlamak için, üretilen KYP hidrojellerin reolojik özellikleri ve bozunma süresi değerlendirildi. iMKH'lerin KYP hidrojelleri içerisine enkapsüle edilmesinden sonra, MTT ve Canlı/Ölü boyama tahlilleri yapıldı. Bu biyomimetik peptit hidrojel iskelelerinin, enkapsüle edilmiş iMKH'ler için uygun bir mikro ortam sağladığını ve geliştirilen KYP hidrojellerinin iMKH'lerin yapışmasını, canlılığını ve çoğalmasını desteklediğini gösterdik. Sonuçlar, üretilen biyoaktif SAP hidrojel iskelelerinin kıkırdak dokusunun yenilenmesini teşvik etmek için yararlı olabileceğini düşündürmektedir.

Anahtar Kelimeler: Kıkırdak doku mühendisliği, Heparin-mimetic, Mezenkimal kök hücreler, Peptit, Kendiliğinden yapılanan peptit hidrojel.

Enhanced Proliferation of Human Mesenchymal Stem Cells by Self-Assembled Peptide Hydrogel Modified with Heparin Mimetic Peptide

Abstract

The development of three-dimensional (3D) microenvironments that imitate the role of natural extracellular matrix (ECM) components is very crucial. Self-assembling peptide (SAP) hydrogels have been utilized as scaffolds for encapsulation, proliferation, 3D culture, and differentiation of cells and also for repairing defects in cartilage tissue. SAPs can be biofunctionalized with short peptide epitopes to form biomimetic scaffolds. Herein, KLD (KLDLKLDLKLDL) SAP was functionalized with a short bioactive motif, EGDK, to fabricate KLD-HM (KLDLKLDLKLDL-EGDK) SAP hydrogel and self-assembled. In the current study, the goal was to explore the effect of developed KLD-HM SAP hydrogels on the viability and proliferation of human mesenchymal stem cells (hMSCs). For ensuring the stability of SAPs, the degradation time and the rheological features of produced SAP hydrogels were assessed. After the hMSCs encapsulation in SAP hydrogels, MTT and Live/Dead staining assays were conducted. We showed that these biomimetic peptide hydrogel scaffolds provided a proper microenvironment for encapsulated hMSCs and the developed SAP hydrogels encouraged the adhesion, viability, and proliferation of hMSCs. The results suggest that produced bioactive SAP hydrogel scaffolds might be useful for encouraging the regeneration of cartilage tissue.

Keywords: Cartilage tissue engineering, Heparin-mimetic, Mesenchymal stem cells, Peptide, Self-assembled peptide hydrogel.

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1. Introduction

Cartilage tissue repair and regeneration is a significant and continuing challenge in regenerative medicine since cartilage is a hypocellular and mostly an avascular, aneural, and alymphatic type of tissue (Arslan, Üstün Yaylacı, Güler, & Tekinay, 2016; Lim, Sardinha, & Myers, 2014). This characteristic of cartilage restricts the ability of the tissue to self-repair. When the cartilage tissue is damaged, the limited spontaneous-repair ability of cartilage becomes inadequate and the regeneration could not take place effectively (Wei et al., 2021). The defects that occurred due to the restricted repairing may cause degenerative arthritis problems (Arslan, Üstün Yaylacı, et al., 2016). Osteoarthritis (OA) is a type of common degenerative disorder of joints, affecting mostly the articular cartilage and the incidence of OA is anticipated to consistently rise over the next decades increases depending on risk factors such as age, sports traumas, obesity, unhealthy diet, and heredity (Chung & Burdick, 2008). Moreover, OA affects both the articular cartilage and the adjacent tissues including the subchondral bone, tendons, ligaments, synovium, and meniscus. Therefore, there is a need for improved treatments to protect, regenerate, and function with full integrity of the cartilage. The current treatment techniques for cartilage regeneration are arthroscopic methods, drilling, microfracture, autologous chondrocyte implantation, mosaicplasty, the implantation of matrix-induced autologous chondrocyte, and osteochondral allograft transplantation (Chung & Burdick, 2008; Wei et al., 2021). Since the disadvantages of existing methods involve morbidity in the donor region, refusal of graft material, complex surgical processes, infection possibility, and the creation of temporary fibrocartilaginous tissue, these techniques might be considered inadequate for long-term tissue reparation, and novel and alternative approaches are required for being capable of successful healing of the cartilage defects (Vinatier & Guicheux, 2016). Tissue engineering provides an alternative treatment method to conventional treatments and offers excessive potential for the regeneration of damaged cartilage by producing biological tissue substitutes such as multifunctional scaffolds.

To imitate the unique structure of cartilage, the use of scaffolds that can ensure structural support, and tissue regeneration is required. Although natural materials are preferred due to the adequate biological features, low cost, and their resemblance to native cartilage extracellular matrix (ECM), their properties such as limited mechanical strength and rapid degradation make these materials disadvantageous (Lum & Elisseeff, 2003). However, synthetic polymer materials which are not bioactive as natural materials, can ensure some required scaffold features such as having tunable physical and chemical properties, producibility with distinct dimensions and forms, having less immunogenic reactions and toxic effects (Arslan, Üstün Yaylacı, et al., 2016; Lum & Elisseeff, 2003). Hydrogels with high water content, biodegradable and biocompatible 3D polymeric, and hydrophilic networks resemble natural tissues (Vedadghavami et al., 2017). The hydrogels as scaffolds in cartilage tissue engineering have attracted intense interest from researchers due to the similarity to the original cartilage ECM and their porous structure (M. Liu et al., 2017). Moreover, hydrogels also offer minimally invasive features due to the capability to fit into irregular-shaped damages (Vedadghavami et al., 2017; Yang, Zhang, Yue, & Khademhosseini, 2017). Self-assembling peptides (SAPs) are biomaterials that might be turned into hydrogels by self-assembling which is a process that provides disordered constituents or molecules spontaneously organize into more regular via certain interplays between them without a need for an outside action (Dikecoglu et al., 2018; Nune, Kumaraswamy, Maheswari Krishnan, & Sethuraman, 2013). Furthermore, synthetic SAP hydrogels have been utilized as the injectable form of biomaterials to repair tissues and modified with bioactive factors or components which can support cell-matrix interplays (Chung & Burdick, 2008). Kisiday *et al*. encapsulated chondrocytes into designed KLD-12 peptide (AcN-KLDLKLDLKLDL-CNH_2, K: Lysine, L: Leucine, D: Aspartic acid) and obtained a 3D hydrogel scaffold structure where chondrocytes retain their morphology. Moreover, cartilage-similar ECM that is rich in proteoglycans and collagen type II content was observed in KLD-12 hydrogels (Kisiday et al., 2002). In another work, KLD-12 hydrogel may work like an injectable scaffold for the field of tissue engineering of the intervertebral disc (Sun & Zheng, 2009). Moreover, Sun *et al.* evaluated the biocompatibility of KLD-12 within animals for tissue engineering of intervertebral discs and reported that KLD-12 hydrogel structure possessed a good biocompatibility feature to the host rabbit and MSCs (Sun et al., 2010). The lack of biological cues on SAPs gives rise to the need for further modification. To functionalize the KLD-12 hydrogels, the incorporation of functional peptide sequences in KLD-12 SAP might be a promising approach for directing and enhancing cellular viability, activity, and functions (Lu & Wang, 2018; Pérez et al., 2015). In recent work, Onak *et al*. modified KLD-12 peptide with functional glutamic acid peptides (EEGGC and EEEEE) for producing KLD-EEGGC and KLD-EEEEE and reported that produced bioactive SAP scaffold induced the human mesenchymal stem cells (hMSCs) osteogenic differentiation (Onak, Gökmen, Yaralı, & Karaman, 2020). Furthermore, Li *et al*. mixed HAVDI which is the N-cadherin-derived peptide, and SAP KLD-12 and stated that the self-assembled hydrogel construct that was functionalized by N-cadherin mimetic peptide increased chondrogenesis of the hMSCs (Li et al., 2017).

In the ECM of articular cartilage, proteoglycans are described as the hydrophilic protein molecules which possess one or more chains of glycosaminoglycan (GAG) molecules covalently linked to the protein core (Carballo, Nakagawa, Sekiya, & Rodeo, 2017). GAGs are significant elements found in ECM of natural cartilage, these molecules supply biological cues to the chondrocytes and stem cells for cartilage tissue's growth and effective regeneration (Yaylaci et al., 2016). Sulfated GAG molecules, involving heparin and heparan sulfate, stimulate the growth factors binding and also ease the growth factor-mediated signaling (Kocabey, Ceylan, Tekinay, & Guler, 2013). Mammadov *et al.* explored the interplays among heparin-mimetic peptide (EGDK) nanofibers and growth factors and reported that heparin mimetic peptide nanofibers show better binding profiles to vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and fibroblast growth factor-2 (FGF-2) than the control group fibers (Mammadov, Mammadov, Guler, & Tekinay, 2012). Herein, heparin-mimetic SAP hydrogels which are able to imitate natural cartilage tissue were developed and the influence of designed hydrogels on the viability of hMSCs was evaluated. To the best of our knowledge, heparin-mimetic peptide-functionalized KLD SAP sequences have not been reported. In this study, first, KLD was modified by heparin-mimetic peptide (EGDK) for obtaining KLD-EGDK peptide and self-assembled for achieving peptide hydrogel

formation. The rheological features and biodegradation analysis of fabricated SAP hydrogels were determined. After the encapsulation of human bone marrow mesenchymal stem cells (hMSCs) in SAP hydrogels, the impact of developed hydrogels on encapsulated cells was observed and evaluated by the MTT test and Live and Dead assay. The results demonstrate that heparin-mimetic SAP hydrogel promotes adhesion, viability, and proliferation of hMSCs, and these biomimetic SAP hydrogels ensured a proper microenvironment for encapsulated hMSCs.

2. Material and Method

2.1. Peptide Synthesis

Peptide synthesis was performed by the chemical agents provided from AAPPTEC (Louisville, KY, USA) by using a peptide synthesizer (AAPPTEC Focus Xi, Louisville, KY, USA). 4-methylbenzhydrylamine (MBHA) resin with a loading capacity of 0.62 mmol/g was used for the synthesis of KLD (Ac-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-NH_2) and KLD-EGDK (Ac-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Glu-Gly-Asp-Lys-NH_2) peptides via 9-fluorenylmethoxycarbonyl (Fmoc) chemistry method (Onak et al., 2018). First, the resin was mixed with N, N-dimethylformamide (DMF) for 30 minutes for swelling the resin and then rinsed with DMF. Fmoc-protected amino acids (2 equivalents), hydroxybenzotriazole (HOBt; 2 equivalents), N, N-diisopropylethylamine (DIEA; 4 equivalents), and O-benzotriazole-N, N, N, N'-tetramethyluronium-hexafluoro-phosphate (HBTU; 2 equivalents) inside DMF were used for amino acids coupling for 3 hours. Then, 20% 4-methylpiperidine in the DMF solution was used for removing the Fmoc-protecting group for a period of 30 minutes. Ninhydrin test was applied to monitor the reactions and to test for the presence of unreacted amines at each stage. Whenever coupling of all the amino acids to the intended peptide sequence was performed, for separating the peptide from resin, trifluoroacetic acid (TFA): H_2O: triisopropylsilane (TIPS) (95:2.5:2.5) was used. Then TFA was evaporated, and the peptides were rinsed with ice-cold diethyl ether. Next, we centrifuged the suspension for discarding the supernatant part. In the last step, the freeze-drying method was used for the lyophilization process of the resulting peptides (Biobase Biodustry Bk-FD10P, Shandong, China).

2.2. Self-Assembled Peptide Hydrogel Fabrication and Characterization

For producing SAP hydrogels, 2% w/v of the KLD and KLD-EGDK (hereafter denoted by KLD-HM where HM means heparin-mimetic) peptides were mixed with deionized water. The peptide solutions were self-assembled by mixing with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES;25-mM) including Dulbecco's Modified Eagle's Medium (DMEM) with no fetal bovine serum (FBS). After the solution was filtered with a syringe filter with 0.2 μm pore size, the solution was placed inside the incubator for the process of self-assembly.

2.2.1. Rheology Analysis

To observe the mechanical features of produced hydrogels, rheology analysis was carried out. Samples having disk shapes were separated from the gel by utilizing a 20-mm cork borer tool and then prepared hydrogels were placed onto discovery hybrid rheometer-2 (HR2, TA Instruments, New Castle, DE). Then the storage (G′) and loss moduli (G″) values of samples were observed and noted with some certain parameters including shear strain (1%), the distance (0.5 cm), and angular frequency (0.1-10 rad/s), as beforehand defined (Barati, Moeinzadeh, Karaman, & Jabbari, 2014).

2.2.2. In vitro Degradation Analysis

In vitro biodegradation analysis of peptide hydrogels was carried out within simulated body fluid (SBF) at 37°C till the specimens were fully degraded. Preparation of the SBF was done via dissolving NaCl (136.8 mM), K_2HPO_4 (1.0 mM), $MgCl_2.6H_2O$ (1.5 mM), $NaHCO_3$ (4.2 mM), KCl (3.0 mM), $CaCl_2$ (2.5 mM), and $NaSO_4$ (0.5 mM) chemicals inside the deionized water (Kokubo & Takadama, 2006). After the preparation of SBF, $NaHCO_3$ (60 mM) was incorporated till the pH 7.4. Then, hydrogel structures with disc shape were continually shaken within an orbital shaker device at 110 rpm inside SBF with 12 mm diameter. After that, hydrogel structures were lyophilized and weighed at specific periods, then losses in their mass were carefully obtained (Barati et al., 2014).

2.3. Cell Culture and Encapsulation in Peptide Hydrogels

hMSCs (HMSC-AD-500, CLS cell lines Service, Lot#102, Eppelheim, Germany) were cultivated in the basal medium (DMEM containing 250 ng/mL fungizone, 10% FBS, 100 μg/mL streptomycin, 50 μg/mL gentamicin, and 100 units/mL penicillin). The medium was changed with fresh medium regularly. Whenever cells reached 80-90% confluency, cells were passaged by utilizing the solution of 0.25% trypsin/EDTA, then hMSCs were taken into a novel flask containing fresh culture media (K. Karaman, Kumar, He, & Jabbari, 2012; O. Karaman et al., 2016; Sendemir-Urkmez & Jamison, 2007). For the experiments, passage 3 cells were utilized for cultivation. Then, hMSCs suspension was encapsulated within hydrogels (5×10^6 cells/mL) in basal medium. They were placed into the incubator and incubated at 37 °C, 5% CO_2, and cultured.

2.4. Cell Proliferation Assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay, Invitrogen, USA) assay was carried out at 1, 4 and 7 days of cultivation, for assessing cell proliferation analysis, based on the reader (Biotek Synergy HTX, Winooski, VT, USA) was used to measure the absorbance values at 570 nm and values were recorded.

2.5. Live and Dead Assay

For observing cell viability of hMSCs, Live and Dead staining assay was assessed at 7. day. Evaluation of cell viability was done via utilizing the Viability/Cytotoxicity Assay Kit for Animal Live and Dead Cells (Biotium, Inc. Hayward, MN,

manufacturer's instructions as it was beforehand explained in a study (Onak & Karaman, 2019). In brief, after removing the cell medium from hydrogels, 10% MTT dye in the culture medium was transferred to hMSCs, and hydrogel structures were incubated during 4 hours at 37°C and 5% CO_2. It is important to note that all MTT procedures were performed in the dark. Later on, the medium was taken from hydrogels and then for dissolving the formed formazan crystals, dimethyl sulfoxide was given. After 10-15 minutes incubation period, a microplate

USA) with respect to instructions of the manufacturer. Briefly, first, the culture medium was removed from hMSC encapsulated hydrogels. PBS was used to wash hMSCs for two times. Then, the viability/cytotoxicity assay solution, including 5 µL Calcein acetoxymethyl ester (Calcein AM;4 mM) and 20 µL Ethidium homodimer III (EthD-III; 2 mM) in 10 mL PBS, was added to hMSCs. Then they were incubated in the incubator during 20-30 minutes at room temperature. Following the incubation, to create fluorescent images of stained cells, well-plate was placed on a fluorescence microscope (Olympus CKX41, Tokyo, Japan). Lastly, live cells (green) and dead cells (red) were stained by Calcein AM and EthD-III, respectively were imaged by the microscope, and images were recorded.

2.6. Statistical Analysis

We conducted our experiments with at least three repetitions. All the obtained data were statistically analyzed with two-way analysis of variance (ANOVA) (SPSS 12.0, SPSS GmbH, Germany) and the Student-Newman-Keuls method as a post hoc test. Significant differences among groups were defined at p values at least less than .05. (*p < .05, **p < .01, ***p <.001).

3. Results and Discussion

3.1. Characterization of Self-Assembled Peptide Hydrogels

The peptide of KLD and KLD-HM were synthesized and KLD and KLD-HM SAP hydrogel groups were produced successfully. Here, the addition of heparin-mimetic peptide at the N-terminus of the KLD peptide did not influence the solubility of SAP in water. Also, the addition of heparin-mimetic epitope to KLD peptide did not influence the process of self-assembly. Hydrogels were characterized based on the rheology and degradation properties. Assessment of rheology measurements was done for comparing the macroscopic features of SAP hydrogels (Figure 1). KLD and KLD-HM SAP hydrogels had similar rheological properties. The maximum storage modulus was found under 1,000 Pa for both groups. It is seen that G' and G" as a function of frequency have stayed relatively constant. The storage modulus (G') of KLD hydrogel was found among 400 and 500 Pa, loss modulus (G") was found between 60 and 80 Pa, and the storage modulus (G') in KLD-HM hydrogel was observed among 400 and 600 Pa, the loss modulus (G") was observed among 40 and 85 Pa. KLD and KLD-HM hydrogels have nearly the same storage and loss modulus values with each other, and there is no significant difference.

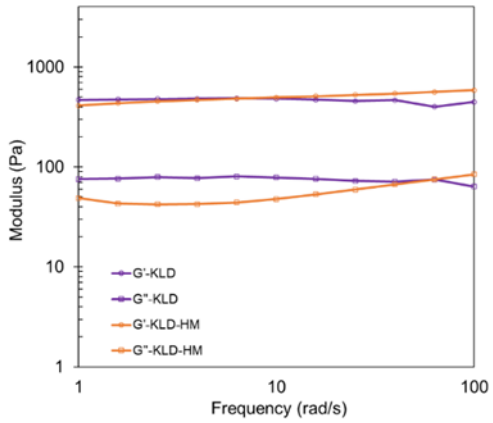

Figure 1. Rheology analysis of KLD, and KLD-HM peptide hydrogels. G' represent storage modulus and G" represent loss modulus.

Şekil 1. KLD ve KLD-HM peptid hidrojellerinin reoloji analizi. G' depolama modülünü ve G" kayıp modülünü temsil eder.

Every 7 days, we calculated the biodegradation analysis of SAP hydrogels (Figure 2). The fully degradation time was found as similar in KLD and KLD-HM hydrogels. Complete degradation of KLD and KLD-HM peptide hydrogels occurred after 63 and 56 days, respectively. There was no statistically significant difference in the degradation time of KLD and KLD-HM hydrogels. During tissue regeneration, for ensuring SAP hydrogel stability, SAP hydrogels' degradation rate, which is defined by the environmental circumstances, should take place in a similar period (Koch et al., 2018). Hydrogels as biomaterials might be utilized in the form of injectable scaffolds and with this form, they can fill any size and shape of defects, and also their implantation might become in minimally invasive way (Chung & Burdick, 2008). For developing a biodegradable hydrogel, Parmar *et al.* combined a Streptococcal collagen-like 2 proteins with hyaluronic acid (HA) or chondroitin sulfate (CS)-binding peptides and after that cross-linked with a matrix metalloproteinase 7 (MMP7)-sensitive peptide and after hMSCs encapsulation, hydrogels showed enhanced viability and remarkably increased chondrogenic differentiation as compared with control which has not modified by GAG-binding peptides (Parmar et al., 2015). Liu *et al.* utilized a collagen mimetic peptide (CMP) with the sequence of GFOGER and modified poly (ethylene glycol) (PEG) hydrogel by GFOGER for using like a scaffold and reported that PEG–CMP hydrogels ensured the native environment that supports hMSCs chondrogenesis and also increases the cartilage-specific ECM secretion when compared with unmodified PEG hydrogels (S. Q. Liu et al., 2010). Moreover, Wu *et al.* intended to explore the implementation of hyaluronan (HA) microenvironment for human adipose-derived stem cells (hADSCs)-based regeneration of articular cartilage tissue. They produced the HA-enriched fibrin hydrogels and reported that the HA microenvironment increases the hADSC-mediated cartilage regeneration within chondral defects (Wu et al., 2018).

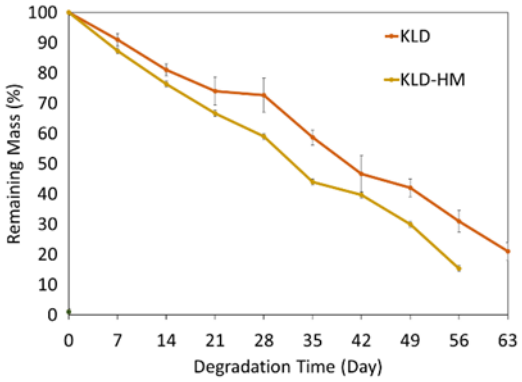

Figure 2. Biodegradation of KLD, and KLD-HM hydrogels. Error bars represent mean ± SE (n = 3).

Şekil 2. KLD ve KLD-HM hidrojellerinin biyobozunumu. Hata çubukları ortalama ± SE'yi temsil eder (n = 3).

3.2. Cell Proliferation of hMSCs in SAP Hydrogels

In current work, it was aimed to assess the influence of heparin-mimetic SAP hydrogel on the viability and proliferation of encapsulated hMSCs. Cell proliferation analysis of hMSCs encapsulated within SAP hydrogels was done using the MTT test on 1, 4, and 7 days of their incubation (Figure 3). In Figure 3, the number of cells on the day of cell encapsulation into hydrogels was taken as 100% and cell viability was calculated as a percentage according to the absorbance values obtained. According to the results from the MTT assay, the cell number for both KLD and KLD-HM increased with incubation time, suggesting that cells encapsulated in peptide hydrogels were capable of maintaining their viability and proliferate. However, the cell number in KLD-HM hydrogel was remarkably greater than in KLD hydrogel at 1, 4, and 7. days. Moreover, these increased cell numbers in both of the hydrogel groups recommended that the SAP hydrogels did not show a toxical impact on hMSCs. These findings emphasized the importance of adding peptides onto SAP hydrogels. Herein, the impact of heparin-mimetic peptide on cell proliferation was assessed with the MTT test and our findings revealed that KLD-HM hydrogels facilitated the proliferation of encapsulated cells and hMSCs were capable of adhering to hydrogel surface and there was sufficient area for cell proliferation. Our results suggest that the presence of heparin-mimetic peptide in KLD hydrogel induces hMSCs proliferation. Molecular self-assembly has become a novel area to develop scaffolds for use in the tissue engineering field because of its ability to imitate the native ECM both structurally and functionally (Nune et al., 2013; Webber, Kessler, & Stupp, 2010). Biomimetic SAPs are important components as building blocks to produce hydrogel scaffolds having 3D network constructions, that might imitate the original ECM (Lu & Wang, 2018). To produce biomimetic scaffolds, SAP hydrogels can be functionalized utilizing bioactive components to provide biological cues for cells, and in that way allow guiding and controlling the cellular behaviors. For such a modification, short peptide epitopes can be used to ensure biofunctionality to SAP scaffolds. In a recent study, KLD SAP was functionalized with bioactive peptide motifs for the regeneration of bone tissue (Onak et al., 2020). Also in another recent study, KLD SAP was modified with laminin-derived peptides for investigating vasculogenesis (Onak Pulat, Gökmen, Çevik, & Karaman, 2021) Cimenci *et al.* used N-Cadherin mimetic peptide (HAV-PA) and self-assembling E-PA [Lauryl-VVAGE] together and produced N-cadherin mimetic peptide nanofibers supported the MSCs chondrogenic differentiation with chondrogenic factors, like a synthetic ECM platform (Eren Cimenci, Kurtulus, Caliskan, Guler, & Tekinay, 2019).

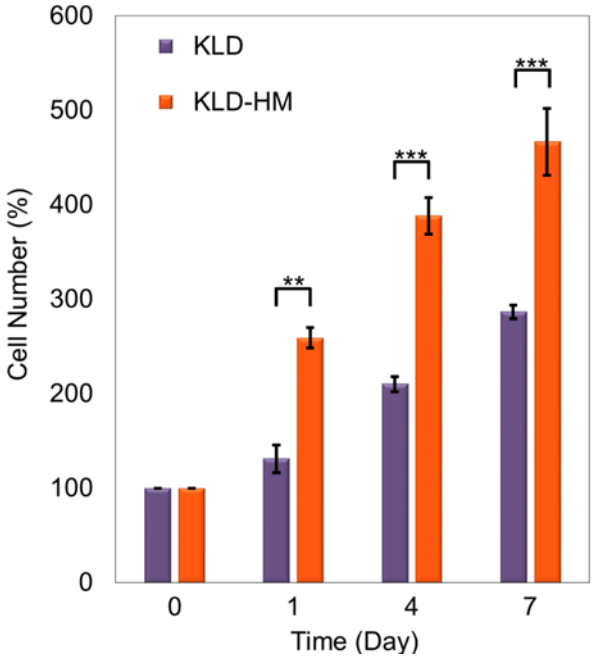

Figure 3. Cell number analysis of hMSCs found in KLD, and KLD-HM hydrogels and incubated in basal medium for 7 days.

Şekil 3. KLD ve KLD-HM hidrojellerinde bulunan ve bazal ortamda 7 gün inkübe edilmiş iMKH'lerin hücre sayısı analizi.

3.3. Live and Dead Staining

Live and Dead assay was done for evaluating hMSCs viability in KLD and KLD-HM hydrogels. Fluorescent microscopy images that were obtained from Live and Dead staining analysis are shown in Figure 4. In these images, live cells are shown with green color and dead cells are shown with red color. These images demonstrated that in both KLD and KLD-HM hydrogels, there were both live and dead hMSCs. However, the intensity of green color was greater for KLD-HM hydrogels compared with that of KLD peptide hydrogels, indicating that KLD-HM hydrogels have a greater number of viable cells and cell viability was superior. Moreover, these results show that our results from cell viability analysis are in line with MTT assay results. Herein, we incorporated a heparin-mimetic peptide epitope to KLD SAP to mimic the native ECM. When their crucial role in tissue remodeling is taken into account, incorporating the GAG-mimetic elements within the 3D platform is a positive approach for osteogenic and chondrogenic differentiation of stem cells (Arslan, Guler, & Tekinay, 2016). Similar to integral constituents of proteoglycan molecules, GAG molecules regulate cell behaviors, like differentiation, adhesion, proliferation, and migration of cells by interplaying with various GAG-binding proteins within ECM structure and also mediating the cell signaling pathways (Jackson, Busch, & Cardin, 1991; Wang et al., 2017). Also GAGs molecular chains include a lot of carboxyl and sulfate groups, therefore negatively charged macromolecules that interplay with proteins via electrostatic forces are formed (Jackson et al., 1991; Wang et al., 2017). Yaylaci *et al.* utilized peptide amphiphile nanofibers to imitate the role of heparan sulfate GAGs found in ECM via adding functional units of heparan sulfate such as hydroxyl, carboxylate, sulfonate groups, to the peptide system for

triggering MSCs chondrogenic differentiation. They reported that MSCs found on GAG-mimetic nanofiber system created cartilage-similar nodules and stored cartilage-specific matrix elements by day 7 (Yaylaci et al., 2016).

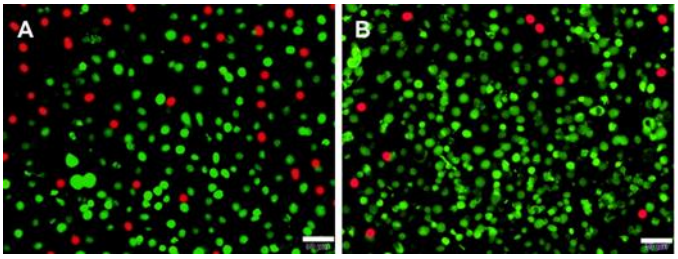

Figure 4. Viability analysis of cells encapsulated in KLD (A), and KLD-HM (B) hydrogels at day 7. Live cells and dead cells are stained with Calcein-AM (green), EthD-III (red), respectively (scale bar represents 50 μm).

Şekil 4. 7. günde KLD (A) ve KLD-HM (B) hidrojellerinde enkapsüle edilmiş hücrelerin canlılık analizi. Canlı hücreler ve ölü hücreler sırasıyla Calcein-AM (yeşil) ve EthD-III (kırmızı) ile boyanmıştır (ölçek çubuğu 50 μm 'yi temsil eder).

Sulfated GAGs like heparan sulfate are able to ease the growth factors immobilization and release with negatively charged sulfate and carboxylate groups of them (Yaylaci et al., 2016). Kocabey *et al*. demonstrated that GAG-mimetic peptide nanofibers having sulfonate and carboxylate groups ensure a proper environment for regeneration and mineralization of bone, and produced nanofibers interplay with bone morphogenetic protein-2 (BMP-2) enhancing the osteogenic cell viability, proliferation, and mineralization (Kocabey et al., 2013). Furthermore, heparin-mimetic peptides have been used to modify tissue engineering scaffolds, since heparin is considered as one of the major integral unit of the GAG molecule. Mammadov *et al.* developed a heparin-mimetic peptide amphiphile molecule that was modified by some biologically active groups to imitate activities of heparin. They reported that these molecules self-assemble for creating nanofibers with the capability for binding to growth factors and for supporting angiogenesis with no requirement for using exogenous heparin or growth factors (Mammadov et al., 2011). In another study, Uzunalli *et al.* carried out a study and demonstrated a heparin-mimetic peptide gel as efficient wound dressing material for functional and also fast repairment of rat full-thickness excisional wound models (Uzunalli et al., 2017). In our study, we used heparin-mimetic peptide (EGDK) for investigating the potential of a heparin-mimetic SAP hydrogel for cartilage regeneration. Based on the performed assays, produced hydrogels were biocompatible and functionalization of KLD with heparin-mimetic peptide promoted the proliferation and viability of hMSCs. Moreover, both MTT analysis and live and dead staining analysis findings revealed that KLD-HM SAP hydrogels provided a good interaction with encapsulated hMSCs. The reason for promising results for KLD-HM hydrogels might be attributed to the idea that these hydrogels presented a better biomimetic surface structure of cartilage tissue ECM, and hMSCs recognized the surface of developed hydrogels due to the presence of heparin-mimetic peptide. Encouraging cell viability and proliferation by the addition of bioactive peptide motifs to synthetic SAPs might be a promising strategy for developing functional scaffolds to be used for cartilage regeneration. Altogether, obtained results propose that heparin-mimetic peptide modified SAP hydrogel could be an ideal biomaterial for cartilage regeneration since they were able to significantly induce cell proliferation and viability by a biomimetic approach. Also, this *in vitro* study reveals that designed KLD-HM hydrogel can be used to trigger cartilage differentiation in future studies and improved further for *in vivo* studies.

4. Conclusion

In summary, we were able to functionalize the KLD SAP with the incorporation of heparin-mimetic peptide epitope, and peptide hydrogel formation was achieved successfully. Heparin-mimetic SAP hydrogel showed improved adhesion, viability, and proliferation of hMSCs when compared against KLD SAP hydrogel. Furthermore, developed KLD-HM SAP hydrogels ensured a proper microenvironment for encapsulated hMSCs in *in vitro*. Therefore, utilizing heparin-mimetic synthetic peptide hydrogels to produce a good microenvironment for hMSCs viability could be thought as a promising approach. The outcomes of the current study reveal that the designed heparin-mimetic SAP hydrogel might have promising potential for mimicking the cartilage tissue and for usage in cartilage tissue engineering applications in the future. Moreover, in the future, it is planned and suggested to carry out chondrogenic differentiation studies with the developed hydrogels. We believed that this study will make a crucial contribution for future cartilage regeneration studies and in the topic of functionalization of biomaterials for promoting the repair and regeneration of cartilage with stem cells.

5. Acknowledge

Authors acknowledge funding from TÜBİTAK (The Scientific and Technological Research Council of Turkey) in the scope of 2209-A University Students Research Projects Support Program and TÜBİTAK 1002 Program (Project No:121S133).

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