PREPARATION AND CHARACTERIZATION OF INJECTABLE ALGINATE-GELATIN BASED TISSUE SCAFFOLDS

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Abstract - Injectable and degradable alginate-gelatin-based scaffolds combined with hydroxyapatite (HAp) and extracted silymarin (ES) were prepared for bone cartilage tissue. Optimization of the gels was made according to swelling, rheological, and mechanical analysis. The effect of adding HAp to the optimum alginate-gelatin gel concentration on swelling, rheological and mechanical properties were investigated. Prepared samples are elastic and mechanically resistant. The addition of HAp decreased the percentage of swelling while decreasing the storage modulus of the hydrogels. Release studies were carried out by adding extracted silymarin to the optimal gel concentration. After 6 hours, 30% silymarin release was observed. Cytotoxicity tests (WST1 and LDH activity test) results showed that alginate-gelatin based hydrogel combined with HAp and ES was non-toxic.

Keywords - Injectable Hydrogel, Alginate, Gelatin, Hydroxyapatite, Silymarin, Tissue Engineering.

I. INTRODUCTION

Aging, joint injury or developmental disorders cause irreversible damage to the articular cartilage. Natural cartilage does not have the blood supply necessary to support self-repair (Ochi et al., 2001). Recently, tissue engineering technology has provided a treatment method for repairing tissue defects and a tissue transplantation technology that promises to solve this problem (Cancedda et al., 2003; Tan et al., 2014). In recent years, unlike scaffolds created outside the body, the ability of injectable scaffolds to adapt to any shape for hard-to-reach areas of the body, regardless of defect geometry, has received much attention. From a clinical perspective, it was reported that the minimally invasive injection procedure can significantly reduce patient discomfort, infection risk, scar formation, hospital stay, and treatment cost (Tan et al., 2014). A gel is needed as a matrix material to produce injectable composites (Burdick and Anseth, 2002). In this process, the gel scaffold is injected in situ to the defective area to cartilage regeneration allowing viable cells to proliferate (LeBaron et al., 2000). Generally, hydrogels are considered as injectable matrix. While forming tissue scaffolds, the ability of the hydrogel mesh to swell by absorbing water provides an advantage by that prevent the collapse of the scaffolds (Yang et al., 2001; Balakrishnan et al., 2005; Rezwana et al., 2006; Fan et al., 2015). There are some important parameters to consider when designing injectable scaffolds. These are toxicity, biocompatibility, mechanical properties, porosity, degradability and in-situ setting (gelling) time of the scaffold material that requires both physical and biological performance (Portnov et al., 2017).

Alginate is widely preferred in various biomedical applications, including wound dressings, dental impression materials, drug administration, and tissue engineering (Sun et al., 2013).To obtain an effective tissue-engineered structure, cells must be released across the scaffold (Woodruff et al., 2012; Stevens et al., 2008). Gelatin contains natural peptide sequences that facilitate cell adhesion and enzymatic degradation.Biomaterials used for bone regeneration should have the feature of osteo-conductivity as well as being biocompatible (Agarwal and García., 2015). Hydroxyapatite (HAp) is frequently used in biomedical applications due to its bioactivity, biocompatibility, non-toxicity, and chemical inertness (Othman et al., 2016; Pighinelli et al., 2014, Bucholz et al., 1989). Silymarin, obtained from the milk thistle plant, has been used as an organic medicine for liver diseases for many years. Although Silybum marianum did not significantly alter the resolution of chronic liver disease, it has been reported to lower liver enzyme levels and exert anti-inflammatory and T-cell modulating effects (Post-White et al., 2007). Also it is reported that silibinin has an effect on bone formation in human bone marrow stem cells (Ying et al., 2013).

In this study alginate-gelatin-based injectable scaffolds were prepared for bone cartilage tissue.Glucono-D lactone (GDL) was used for gelation agent. Silymarin was extracted from the milk thistle plant. The appropriate gel composition was determined according to gelling time, mechanical and swelling properties of gels by changing the amount of gelatin, crosslinkers, and HAp. The effects of HAp on gelation time, swelling, mechanical strength, degradation were investigated. Also silymarin relase and cytotoxicity (WST-1) of the optimum gel was investigated.

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Sodium alginate (SA, with a medium viscosity, 1500cPc. Alfasol) and gelatine (GE-Alfasol) were used to prepare injectable gel. Calcium carbonate

($CaCO_3$, Sigma Aldrich) was used as crosslinker of SA, glutaraldehyde %50 (GA-Acar Chemicals) and (3-Glycidyloxypropyl)trimethoxysilane (GPTMS) (Sigma-Aldrich) were used as crosslinker of gelatine. Glucono-D-lactone(GDL, Sigma-Aldrich) was used as gelation agent. Hydroxyapatite (HAp-Sigma Aldrich) were used to increase both the osteoconductivity and the mechanical strength of the gels. Previous study (Nahirci vd. 2021), silymarin powder was extracted from the milk thistle plant with extraction 7.3% yield by applying solvent extraction method. Silymarin content of silybum marianum seed was found to be 4.21% based on the extracted amount.

Sodium alginate was dissolved in deionized water at a final concentration of 1.5%. Various amount of $CaCO_3$ (0.5, 0.75, 1, 1.5 % (wt/V) and GDL (0.25, 0.5, 0.75, 1, 1.5 % (wt/V) was first dispersed in deionized water than added to the sodium alginate gel. SA gels were mixed well with magnetic stirrer and poured into 24-well plates. After 1 hour gelation time, 3D cylindirical shaped (1cm in diameter x1.5 cm height) gels were obtained. The SA/GDL (V/V) ratio was determined as 5/1 according to the results of the swelling tests in the preliminary experiments. Optimum SA gel concentration (SA/$CaCO_3$/GDL: 1.5/1.5/0.75) was found according to swelling, rheological, and mechanical tests. Various concentration GE (0.01, 0.05, 0.1, 0.25, 0.5, 0.1 wt%/V) and GA (0.0188, 0.1, 0.2, 0.5, 0.1wt%/V) was added to optimum SA gel. Optimum SA-GE gel concentration (SA/$CaCO_3$/GDL/GE/GA: 1.5/1.5/0.75/0.5/0.1) was found according to swelling, rheological, and mechanical tests. By adding different amount of HAp (0.25, 0.5, 0.75, 1 wt/V) to the optimum SA/GE gel concentration, the appropriate gel concentration (SA/$CaCO_3$/GDL/GE/GA/HAp:

1.5/1.5/0.75/0.5/0.1/0.75) was found according to swelling, rheological and mechanical tests. Extracted silymarin (ES) (0.1 wt%/V) was then added to SA/$CaCO_3$/GDL/GE/GA/HAp gel. Compositions of hydrogels are given in Table 1.GA, one of the cross-linkers of gelatin, was used in gel optimization due to its cheapness, and FDA-approved (Gull et al., 2020) GPTMS was used in the same amount as GA in cytotoxicity experiments. The pH value of prepared hydrogels was monitored by pH-meter (Ohaus-Starter 3100M) during gelation within 1 h at room temperature.

Swelling behavior of the hydrogels was studied in PBS with 1 mM $CaCl_2$ at 37°C. The swelling ratio was defined as $(W_s - W_d)/W_d$. The swollen gel was weighed after being lyophilized (W_d) and the following wet weight (W_s) during swelling experiment (1st day of gelation) was recorded after the excess water on the surfaces was absorbed by filter paper.

Morphologies of the samples were investigated by SEM (Fei Quanta Feg 250) after being lyophilized, sliced and platinum-coated. Crystal structure were investigated by XRD (Bruker D8 Advance Twin-Twin). Using Ni-filtered Cu-Kα radiation, measurements were made at a scanning speed of $6^{\circ}min^{-1}$, with an angle of 2θ between 5 and 70°.

No	SA %wt/V	$CaCO_3$ %wt/V	GDL %wt/V	GE %wt/V	GA %V/V	HAp %wt/V	ES %w/V
1	1.5	0.5	0.75	-	-	-	-
2	1.5	0.5	0.75	0.05	0.1	-	-
3	1.5	0.5	0.75	0.05	0.1	0.25	-
4	1.5	0.5	0.75	0.05	0.1	0.5	-
5	1.5	0.5	0.75	0.05	0.1	0.75	-
6	1.5	0.5	0.75	0.05	0.1	1	-
7	1.5	0.5	0.75	0.05	0.1	0.75	0.1

Table1: Composition of hydrogels

Rheological analysis of hydrogels were made by using a rheometer (Haake Mars II) with a parallel plate (PP25). The prepared gel mixtures were poured onto the rheometer bottom plate and the evolution of storage (G′) and loss (G″) modules was recorded as a function of time at a constant frequency of 0.5 Hz and a constant strain of 0.05. The gelation time, as a kinetic parameter for network formation, is defined as the transition time between G′ and G″. The gelation time was determined from the intersection points of G' and G' (Tan, H., et al. 2014).Gel scaffolds injected into bone defects encounter cyclic forces. Angular frequency sweep was conducted on samples to demonstrate the stability of the three-dimensional crosslinking network in the gel system under shear stress. Samples were fabricated in dishes and cut into thin disks (30 mm diameter, 2 mm height). The angular frequency sweep was performed by varying the angular frequency from 0.1 to 100 rad/s at strain of1%. Storage modulus (G′) and loss modulus (G″) were recorded.

Mechanical tests were performed on cylindirical shaped gels (3cm in diameter x3cm height) according to ASTM D-1621 with a force of 5kN and a compression speed of 2.5mm/min by using TA- XT

Plus Texture Analyser. The reported results are the average values of three measurements.
Degradation study was conducted using PBS with 1 mM $CaCl_2$during 28 days. Samples were weighed (W_i) and then placed in plastic tubes filled with 15 mL PBS solution and incubated at 37°C. Samples were withdrawn and weighed (W_t) at the end of each degradation cycle (1, 2, 3, 4, 7, 14, 21 days). Mass loss percent was defined as $(W_t\text{-}W_i/W_f)x100$. The reported results are the average values of three measurements.

In order to determine the time-dependent release amounts of silymarin loaded samples, release experiments were performed in PBS solution with 1 mM $CaCl_2$at 37°C. The samples were immersed in 100mL of PBS solution in a shaking bath, 4 mL of sample was taken at certain time intervals (1, 2, 3, 4, 5, 6 hours) and 4 mL of fresh PBS solution was added to make up to 100 mL again. The samples were centrifuged, separated from the supernatant and kept for UV analysis. Silymarin release was also conducted in degradation experiments. UV-analysis was performed with a Perkin Elmer Lambda25 spectrophotometer at 288 nm wavelength.

The cytotoxicity and cell viability of the SA/$CaCO_3$/GDL/GE/GPTMS/HAp/ES gel was determined according to the cell proliferation reagent WST-1 protocol. Human fibroblast cells (P5) were obtained from the stem cell development center (KOGEM) cell collection. Cells were established by culturing for 24-72 hours in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco, Thermo Scientific, Paisley, UK) and 1% Penicillin/Streptomycin (Gibco). 100,000 cells were seeded on hydrogel-coated and uncoated surfaces in 24-well polystyrene culture dishes and incubated at 37°C, 5% CO_2, 100% humidity (saturated).Cell viability was measured by the WST-1 assay (Roche, Mannheim, Germany). The test was performed according to the manufacturer's instructions. Briefly, at the time of measurement, the medium in the culture dishes was removed and 10% (10% WST-1 reagent, 90% RPMI 1640 basal medium) WST-1 solution was added to each well. It was incubated for 45 minutes in a 37°C 5% CO_2 incubator. At the end of the period, 100 µL samples were taken and added to 96-well plates. The color change caused by living cells was determined by measuring at 450nm in a spectrophotometer (VersaMax, Molecular Devices).
The same method was used for the cell adhesion assay. After 24 hours of culture, the surface was washed with PBS and the cells on the surface were determined with WST-1. Cells growing on the uncoated culture surface were used as controls. Results are expressed in % relative to control. The measurement of the samples was repeated at least three times and the results were given by calculating the mean and standard deviation. One-Way ANOVA multiple comparison (one-way ANOVA (post hoc) test) was performed using the SPSS 10.0 program for the analysis of variance regarding the analysis results. If the p-value obtained at the end of the calculation was less than 0.05 ($p<0.05$), the results were considered to be statistically significant.

Determination of the toxic effect on cell viability was performed in accordance with the kit procedure using the lactate dehydrogenase (LDH) plus kit (Roche, Mannheim, Germany). Fibroblasts were seeded on coated 24-well culture dishes using RPMI 1640 (normal) medium and measurements were made after 24, 48 and 72 hours. A sample of 50 µL of broth of the cultured cells was taken and mixed with 50 µL of the reaction solution in a 96-well culture dish. It was incubated for 20 minutes in a 37°C, 5% CO_2 incubator. At the end of the time, the reaction was stopped by adding 50 µL of stop solution. Toxicity was determined according to positive and negative controls by measuring at 490 nm in the spectrophotometer. Toxicity is expressed as percentages. 0% toxicity is the value of cells proliferating in fresh medium without any additives, and 100% toxicity is defined as enzyme activity that occurs when membrane integrity is completely destroyed.

III. RESULTS AND DISCUSSION

3.1. pH value, swelling behavior, morphology and crystal structure of hydrogels

The measured pH value of prepared hydrogels during gelation within 1 h at room temperature are given in Figure 1. Addition of HAp increased the initial pH of the gels. Addition of GDL resulted in a decrease in pH in all gels after 1 hour (range 7.10-7.20).

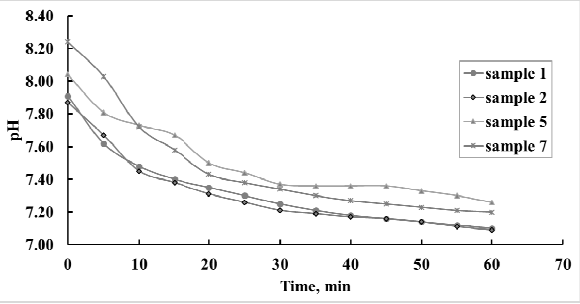


Fig.1. pH value of prepared hydrogels during gelation within 1 h.

Swelling behavior of prepared hydrogels is given in Figure 2. The swelling value of SA hydrogel (sample 1) was observed as 36%, the swelling value of SA/GE hydrogel (sample 2) was observed as 34%. Increasing the amount of HAp decreased the percentage of swelling. The addition of silymarin (sample 7) also slightly reduced the swelling percentage of the hydrogels. Morphologies of freeze dried after swelling experiment SA, SA/GE, SA/GE/HAp and SA/GE/HAp/ES hydrogels, were observed by SEM.

It is seen that all of the prepared samples interconnected and have a porous structure. It can be said that HAp is homogeneously dispersed in the gel (Fig 3-c). Addition of HAp and ES reduced the pore size. XRD patterns of the samples are given in Figure 4. The wide amorphous peak observed at 9 to 17 degrees at 2 theta angle belongs to alginate and gelatin, the observed $CaCO_3$ peaks (JCPD card no: 47-1743)(Wong and Ang 2018), crystal phases defined after HAp addition mostly correspond to HAp (JCPD) card no, 9-0432) (Chaudhuri et al., 2013). The crystal structure of the extracted silymarin was reported as a broad amorphous peak observed at 9 to 17 degrees at 2 theta angle in a previous study (Nahirci et al., 2021).

3.2. Rheological and Mechanical Analysis

The storage modulus is an indicator of the hydrogel's ability to elastically store the deformation energy. It is also directly related to the degree of crosslinking. The higher the degree of crosslinking, the greater the storage modulus. Swelling is also directly related to the degree of crosslinking, high crosslinking restricts swelling. Figure 5 shows storage (G′) and loss (G″) modules of sample 7 versus a function of time at a constant frequency of 0.5 Hz and a constant strain of 0.05.The gelling time at the optimum gel concentration (sample 7) was 317 sconsidering the second point of intersection of G′and G″. The presence of 2 intersections suggests that the crosslinking reaction is continuing.

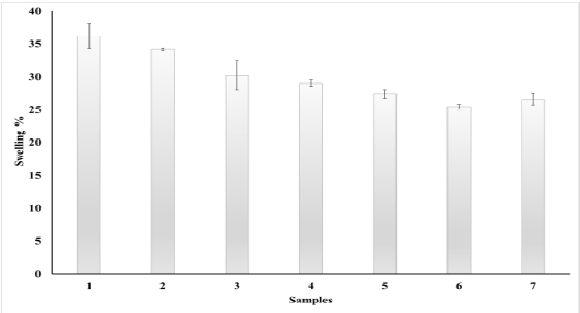


Fig.2. Swelling behavior of prepared hydrogels.

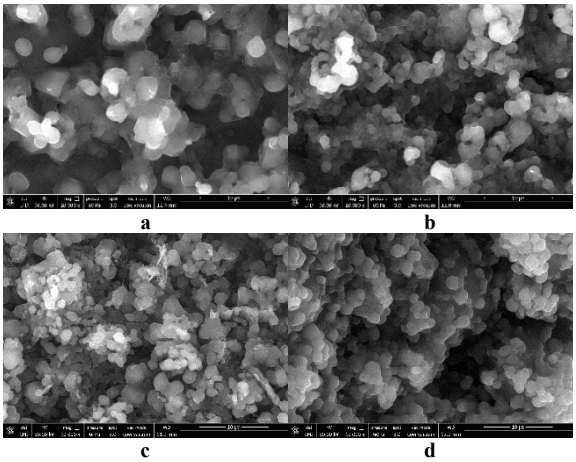


Figure 3. SEM images of a-sample1, b-sample 2,c-sample 5, d-sample 7.

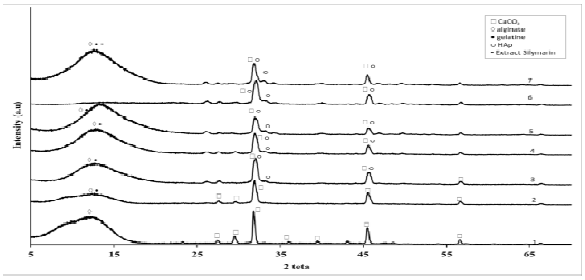


Figure 4. XRD pattern of the prepared samples.

The gelation time of samples were determined from constant frequency sweep tests of the intersection points of G' and G″ and are given in Figure 6. Gelation time was increased by the addition of gelatin whereas decreased by the addition of HAp and silymarin.

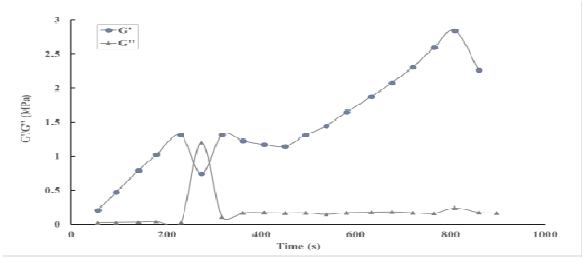


Figure5.Storage (G′) and loss (G″) modules of sample 7 versus a function of time at a constant frequency of 0.5 Hz and a constant strain of 0.05.

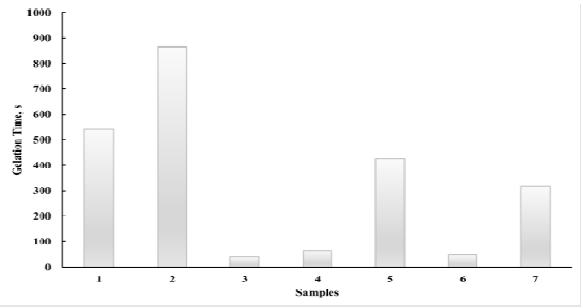


Figure 6 Gelation time of prepared samples

The stability of the three-dimensional crosslinking network in the gel system under shear stress is shown in Figure 7. Higher G' denotes more solid-like property (higher strength or mechanical rigidity). G" denotes liquid-like property.If G' is higher than the G", the material can be regarded as mainly elastic, i.e. the phase shift is below 45°. It was observed that the G'is greater than the G"in all samples. It can be said that the prepared samples are elastic and mechanically resistant. The addition of HAp decreased the percentage of swelling and also the G'of the hydrogels.

The mechanical test results of the samples are given in Figure 8.The elastic modulus decreased from 25.6 MPa to 23.44 MP with the addition of gelatin and HAp. Yield stress decreased from 1.2MPa to 0.73 MPawith the addition of gelatin and HAp.After the mechanical test, no breakage was observed in the

samples as seen in Figure 9 however a slight decrease in their height was observed.

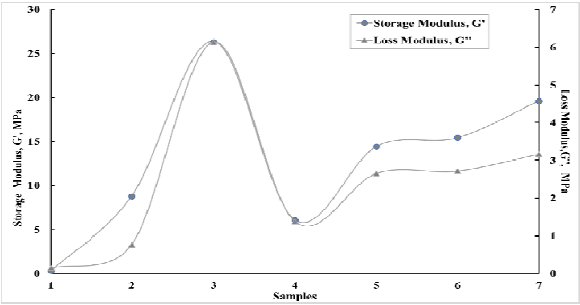


Figure 7. Storage (G′) and loss (G″) modules of prepared samples by angular frequency sweep from 0.1 to 100 rad/s at strain of1%.

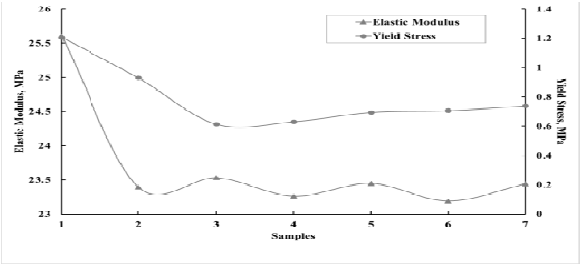


Figure 8. Mechanical properties of samples.



Figure 9. Image of samples after mechanical test.

3.3. Degradation

Degradation experiment was conducted in PBS medium with 1 mM $CaCl_2$at 37°C during 21 days. According to Figure 10, the addition of gelatin caused the gels to swell in PBS medium. The addition of HAp reduces swelling and aids degradation. 20% mass loss was observed with the addition of silymarin (sample 7) HAp and silymarin prevent the gel from swelling by disrupting the gel network.

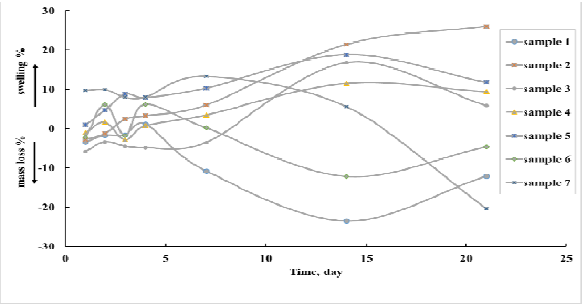


Figure 10. Weight Loss of samples in PBS medium at 37°C versus time

3.4. Drug Release

The results of silymarin release in PBS medium at 37°C are given in Figure 8. The cumulative silymarin release in the first 6 hours is 30%. At the end of 21 days, 92% silymarin release was observed.

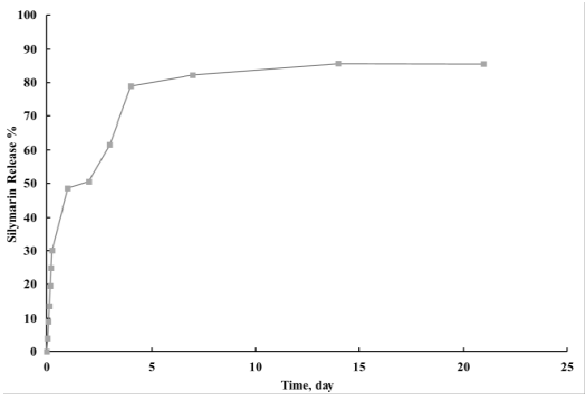


Figure 11. Percent silymarin release plot.

3.5. Cytotoxicity and LDH activity

The cytotoxicity analyzes of the sample 7 were first investigated by the WST1 method on its effect on viability and cell proliferation, and then its effect on cell death by lactate dehydrogenase (LDH) activity measurement.Cells were incubated on the hydrogel for 72 hours, and the proportions of cells adhered to the surface by WST1 every 24 hours were determined by WST1. In general, the proliferation of cells on the hydrogel was not restricted and continuous growth was supported. Compared to the plastic surface, it was observed that the cell proliferation was 3.74 times ($P<0.05$) (Figure 12). However, at the end of the first 24 hours, it was measured that there were fewer cells on the hydrogel. This is related to the cells attached to the surface, and at the end of 24 hours, the number of cells attached to the surface of the hydrogel material was determined to be 3.35 times less than the cells attached to the plastic surface. Cells multiplied rapidly on the improved material in the process following the adhesion.

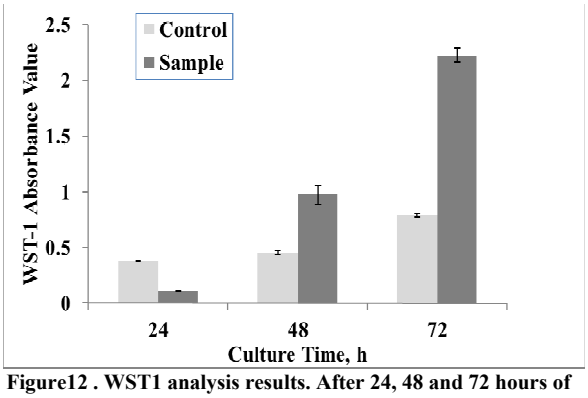


Figure12 . WST1 analysis results. After 24, 48 and 72 hours of culture, the number of fibroblast cells adhered to the surface was determined by WST1. In the control group, cells were cultured on the plastic surface. One-way ANOVA (post hoc) test was used in the analysis of variance between groups. P value (2.69×10-14) was calculated less than 0.05 (P<0.05).

LDH analysis was performed to investigate whether the cause of the decrease in the cell number within 24hrs was as a result of toxic effect. According to the LDH analysis results, the toxicity value was measured to be below 1% at the end of 24 hours (Figure 13). The toxic values of the cultures of cells on the hydrogel (sample) and plastic surface (control) were measured to be 0.63% ±0.15 and 0.22% ±0.05%, respectively. In the following days, the toxicity values increased in parallel with the proliferation. However, this rate remained below 5% at the end of 72 hours. According to these results, although the LDH values increased rapidly in the hydrogel culture, it was determined that it was not toxic. The reason for this rapid increase in LDH value is associated with high cell proliferation.

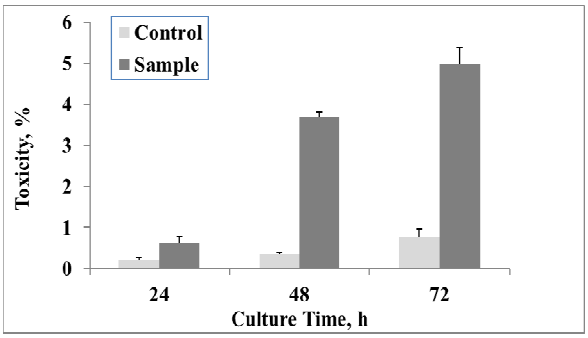


Figure13. LDH activity measurement results. After 24, 48 and 72 hours of culture, LDH enzyme activity was measured in broth containing fibroblast cells in culture. In the control group, cells were cultured on the plastic surface. One-way ANOVA (post hoc) test was used in the analysis of variance between groups. P value (6.44×10-9) was calculated less than 0.05 (P<0.05).

IV. CONCLUSIONS

Injectable and degradable alginate-gelatin scaffolds combined with HAp and silymarin have been successfully prepared for bone cartilage tissue. Although the addition of HAp and gelatin slightly reduced the elastic modulus and yield stress, it increased its resistance against cyclic forces. Cell viability and toxicity were measured by using WST1 and LDH analysis respectively.

It was determined that the hydrogel surface did not support cell adhesion as much as the plastic surface. However, at the end of 48 hours, cell proliferation occurred rapidly with the increase in adhesion. It is known that measurement methods such as WST1 and MTT gave higher results with an increase in the metabolic rate of the cell. From this point of view, it can be said that the additives in the hydrogel have an effect on accelerating the cell metabolism.

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