A Novel Designed Chitosan Based Hydrogel Which is Capable of Consecutively Controlled Release of TGF-Beta 1 and BMP-7

TGF-Beta 1 ve BMP-7'nin Ardışık ve Kontrollü Salımına İzin Veren Yeni Kitosan Bazlı Bir Hidrojel Dizaynı

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ABSTRACT Objective: Both Bone Morphogenetic Protein 7 (BMP-7) and Transforming Growth Factor Beta 1 (TGF-β1) have stimulating effects on chondrocyte proliferation. These growth factors act at different time points of the biological repair process. We aimed to design a hydrogel, which would enable consecutive controlled release of the growth factors. **Material and Methods:** We designed a chitosan particle impregnated Poly (vinylalcohol) (PVA)-borax hydrogel (CPBH). CPBH allowed controlled release of BMP-7 and TGF-β1 at different time points. Hydrogel structure was analyzed by Scanning Electron Microscope (SEM). The release kinetics of Growth Factors (GFs) were determined with enzyme-linked immunosorbent assay (ELISA) and UV-spectrophotometer. Chondrocyte viability and toxicity were tested through CellTiter 96® Non-Radioactive Cell Proliferation (MTS) ELISA. **Results:** The designed hydrogel showed high swelling and mucoadhesion characteristics under acidic conditions. CPBH released BMP-7 first and rapidly and TGF-β1 consecutively and slowly. It also allowed controlled release of protein/peptide based drugs for 21 days without altering their bioactive properties. At the end of 21 days, 82.62% of BMP-7 and 98.34% of TGF-β1 were consecutively released. The difference between the groups was significant (for TGF-β1, $p<0.05$ and for BMP-7, $p<0.001$). **Conclusion:** The controlled and slow release of growth factors has been shown to be beneficial on cartilage regeneration. As it is not cytotoxic, we suggest that this hydrogel might be used in medical and pharmaceutical applications areas.

Key Words: Delayed-action preparations; hydrogels; TGF-β1 protein, human; bone morphogenetic protein 7

ÖZET Amaç: Kemik Morfojenik Proteini-7 (BMP-7) ve Transforme edici Büyüme Faktörü Beta-1 (TGF-β1)'in kıkırdak hücreleri üzerinde prolifere edici etkileri bulunmaktadır. Bu büyüme faktörlerinin etkisi biyolojik tamir sürecinin farklı zamanlarında gerçekleşmektedir. Bu çalışmada, büyüme faktörlerinin ardışık ve kontrollü olarak salınımını gerçekleştirecek bir hidrojel tasarlamayı amaçladık. **Gereç ve Yöntemler:** Bu amaçla kitosan emdirilmiş polivinil alkol (PVA)- boraks hidrojeli (CPBH) tasarlandı. Bu hidrojel BMP-7 ve TGF-β1'in farklı zamanlarda kontrollü salınımını sağladı. Tasarladığımız hidrojelin yüzey yapısı taramalı elektron mikroskobu (SEM) ile değerlendirildi. Büyüme faktörlerinin salım kinetiği enzim aracılı immün deney (ELISA) ve ultraviyole (UV)-spektrofotometre ile saptandı. Kondrosit canlılığı ve toksisite testi "CellTiter 96® Non-Radioactive Cell Proliferation (MTS)" ELISA yöntemiyle değerlendirildi. **Bulgular:** Tasarlanan hidrojel, asidik ortam koşullarında büyük oranda şişme ve mukoadezyon özellikleri sergiledi. CPBH, önce hızlı bir biçimde BMP-7'nin, sonra da yavaş olarak TGF-β1'in salınımını ardışık olarak gerçekleştirdi. İçerisindeki protein/peptit yapısındaki büyüme faktörlerinin biyoaktif özelliklerini değiştirmeksizin, 21. güne kadar kontrollü bir şekilde salınım yapılmasını sağladı. Yirmi birinci günün sonunda, tasarlanmış olan bu hidrojelin, BMP-7'nin %82,62, TGF-β1'in ise %98,34 oranlarında ardışık olarak salınımını gerçekleştirdiği gözlendi. **Sonuç:** Büyüme hormonlarının ardışık ve kontrollü salınımının kıkırdak rejenerasyonu üzerine olumlu etkileri olduğu bilinmektedir. Kondrositler üzerinde herhangi bir toksik etkiye neden olmadığı gösterilen CBPH, medikal alanda ilaç taşıyıcı sistemi olarak önerilebilir.

Anahtar Kelimeler: Gecikmeli hazırlıklar; hidrojeller; TGF-β1 protein, insan; kemik morfogenetik proteini 7

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Polymeric hydrogels have been investigated as biomaterials suitable for carrying drugs with protein and peptide structure to the targeted area without losing their bioactive features.[1] The targeted features of such biomaterials are biodegraded after providing necessary support for the cells in the areas in which they are implanted and not creating toxic effect through this process. In order to provide cell proliferation and differentiation, various growth factors have been placed into these biomaterials called scaffold.[2] These growth factors are known for their release kinetics and effects on the targeted tissue.[3]

TGF-β1 and BMP-7 from the growth factor family are biosignals which feature in cartilage repair whose effect mechanisms are known.[4] TGF-β1 is known to cause fibrosis, hypertrophy and osteophyte development when it is released to the environment for a long time in an uncontrolled way.[5,6] In the end, the cartilage tissue to be obtained will be fibrotic rather than hyaline. The prevention of this late adverse effect may be possible through novel drug delivery systems to be developed. Based on this assumption, we aimed to design a hydrogel, which would allow growth factors to reach the targeted area effectively in a consecutive and controlled way within the desired time period without losing their bioactive features and creating toxic effect on chondrocytes. The second aim of the study was to observe the effect of the controlled release of the GFs on chondrocyte proliferation.

MATERIAL AND METHODS

MATERIALS

Medical grade Chitosan (CS) with a deacetylation degree of 91% was obtained from Biosyntech Inc. (Laval, QC, Canada). PVA with an average molecular weight (Mn) of 78400 g/mol was purchased from Vassar Brothers Medical Center (New York). Borax (Na2B407* 10 H20) (di-sodium tetraborate decahydrate) was obtained from Merck Darmstadt, Germany. Collagenase type II was purchased from Invitrogen Corporation, USA (1 mg/ mL). Megacell RPMI - 1640 medium (1X), fetal bovine serum, penicillin-streptomycin, TGF-β1 and BMP-7 were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Insulin, human transferrin and seleneous acid (ITS premix solution) were obtained from Sigma Saint Louis, Missouri, USA; Dulbecco's Modified Eagle's Medium (DMEM) 1000 mg glucose/L, from Sigma Chemical St. Louis, USA; Sodium Dodesyl Sulphate 10% (SDS) L4522, from Sigma Aldrich, Germany, and Albumin bovine fraction V 048K1652, from Sigma-Aldrich Chem, Germany.

METHODS

After approval of the Ethical Committee of Namık Kemal University (29.07.2010/06-06), a hydogel was designed and was analysed at Molecular Biology and Genetic Research Laboratory of Namık Kemal University. All experiments were performed three times.

2.2.1 Study Design and Preparation of CPBH

Study Design

CPBH was fabricated as an empty hydrogel, TGF-β1 loaded hydrogel and BMP-7 loaded hydrogel. Dual-layer hydrogel was fabricated also to release both GFs consecutively. A 24- well plate (6x4) was used for tests. Each plate was arranged in mainly 2 groups; initial 9 wells were free of cells where chondrocytes were seeded on the last 9 wells (Figure 1). Totally 9 plates were used in the study; 4 plates for release kinetics of GFs, 4 plates for MTS

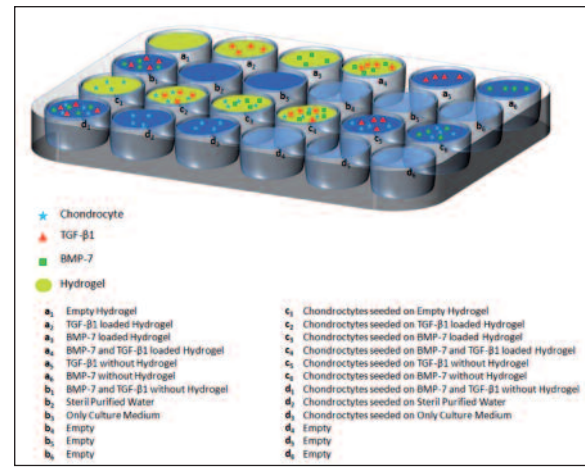

FIGURE 1: Experimental design, the contents of 24-well plate.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

Elisa tests, and 1 plate for scanning electron microscope (SEM) analyses.

Preparation of Empty CPBH

Aqueous solution of PVA 9% (w/v) was prepared by dissolving PVA in ultra pure water in an autoclave (121°C, 1 hour). A chitosan solution 2% (w/v) was prepared by dissolving 200 mg of chitosan in 9 ml of hydrochloric acid (HCl) solution (0.1 mol/L) and was sterilized in an autoclave (121°C, 10 minutes). The chitosan solution was added in small portions to the PVA solution on the heated magnetic shaker device within 1 hour. The chitosan impregnated PVA solution was stirred until cooled to the room temperature to prevent aggregation of the polymers. Saturated borax solution (18%) was prepared with heated magnetic shaker. Finally, borax solution was added to the chitosan impregnated PVA solution in a proportion of 1:3 on the magnetic shaker device, thus crosslinking of the molecules was achieved. Thus, the low hydrophilic capacity of chitosan impregnated PVA solution was improved by saturating the hydroxyl-groups with borax (Figure 2).

Preparation of TGF-β1 loaded CPBH

For this purpose, dry powder form of 10 ng/ml TGF-β1 was separately dissolved in 0.4 molar HCl and 10 mg bovine serum albumin (BSA) and was homogenized with magnetic shaker according to the supplier's instructions.

TGF-β1 (10 ng/ml), was added to the chitosan impregnated PVA solution on the magnetic shaker device before crosslinking the gel with borax. Loaded hydrogel was kept in vacuum-dryer until constant weight was reached.

Preparation of BMP-7 loaded CPBH

BMP-7 (22.7 ng/ml) loaded CPBH was also fabricated as described above.

Preparation of Dual-layer CPBH

The empty, TGF-β1 loaded and BMP-7 loaded hydrogels were cut with a standard cylindrical mold (The hydrogels, which were used to prepere dual-layer hydrogel, were loaded with GFs in an amount achieved after the necessary ratio calculations). The mass of each sample were 1.1 gr.

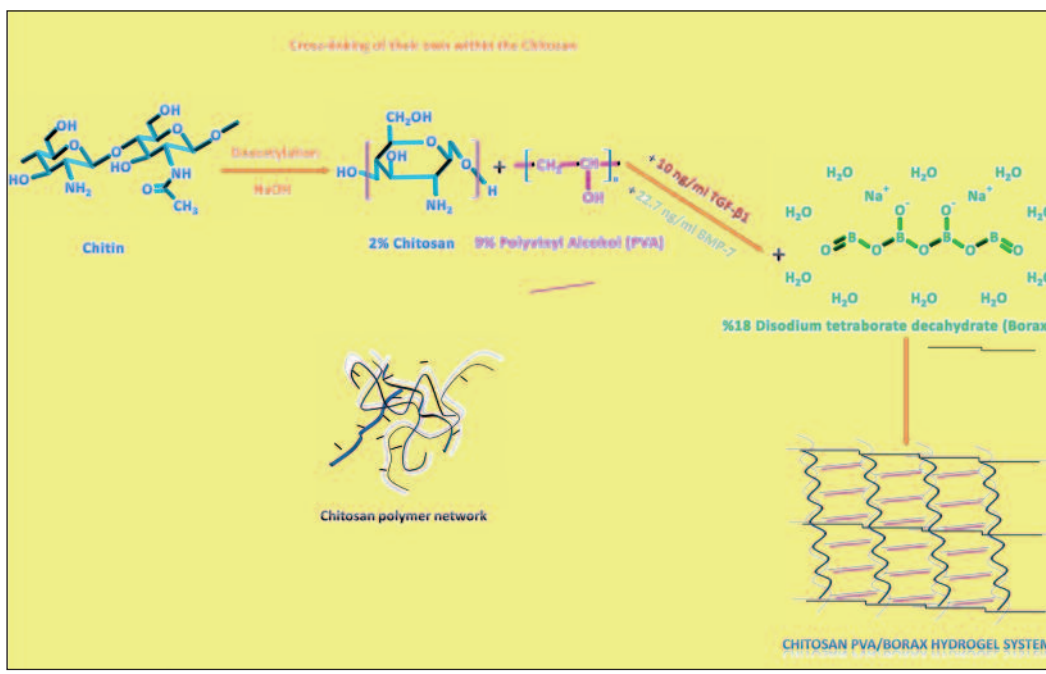

FIGURE 2: Fabrication of chitosan-poly-borax (CPB) hydrogel.
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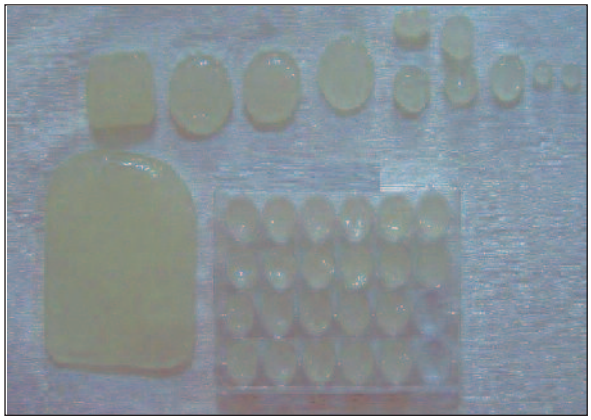

FIGURE 3: Experimental setup.
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TGF-β1 loaded hydrogel was coated with BMP-7 loaded hydrogel and a dual-layer hydrogel was formed, achieving a sequential controlled release pattern with initial BMP-7 followed by TGF-β1.

Each sample was settled on the wells according to the study design (Figure 1, 3). Air bubbles in the solutions were removed by settling them at room temperature. They were stored at -20°C for 6 hours and then were stored at room temperature (22.4°C) for 6 hours.

Characterization of Hydrogel

Scanning Electron Microscopy (SEM) Analysis

Porous structure of CPBH was investigated by SEM analysis. Size analysis and surface area measurements were performed. One additional sample for each group was prepared for SEM analysis. Hydrogels were kept at -20°C to freeze and left to freeze-dry (Thermo Scientific, Savant Modulo Freeze Drying Systems, and USA). Dry samples were coated with 5 nm silver with Sputter Coater (Baltec SCD 005).

Equilibrium Swelling Test

Hydrogels were divided into 4.5 mm inner diameter glass tubes in 1.5x10 mm pieces. These pieces were frozen under -20°C for 6 hours and were further kept in 22.4°C for an additional 6 hours. Dry weights of these pieces were measured and were recorded (W1). These hydrogels were separated from the media by filtration and were inserted into the 45 ml ultra pure water containing glass beakers for 240 minutes. They were brought out from the beakers in every 20 minutes, their surfaces were dried with a blotting paper and they were separately weighed (W2). Dynamic weight and swelling index (hydration percentage) were calculated according to the following formula.[7,8]

Temperature based alteration of diameters test

$$\% \text{ Dynamic weight changes (hydration percentage)} = \frac{W2 - W1}{W1} X\ 100$$

Diameter measurements and their averages were taken with a digital calliper, in 25ºC and 60°C separately. Each measurement was performed three times.

pH based alteration of diameters test

Diameters of hydrogels were further measured in ultra purified water at pH values of 6.0/6.5/7.0/7.4/8.0, and their averages were taken. Each measurement was performed three times.

Prediction of the release kinetics of CPBH

In order to predict the controlled release kinetics of our main GFs, we undertook a control analysis. This analysis was performed before ELISA by measuring absorbance/time values of BSA hydrogel under 280 nm with UV-spectrophotometer.

The BSA (2 mg/ml) loaded hydrogels were immersed in 0.1M sodium phosphate buffered saline (pH 7.4 containing 0.145M NaCl) in a conical flask and were incubated at 37.4°C by shaking. First, the absorbance of CPBH containing BSA and empty CPBH was measured while the phosphate buffer was blank. Then the absorbance of CPBH containing BSA was also measured while the empty CPBH was the blank. All the measurements were done for seven days each being at the same hour, four times a day at 37.4°C. The averages of both were calculated. All the measurements were done three times and were performed at 37.4°C and pH=7.4 phosphate buffer solution. Molar concentration was calculated by the formula (A/ε)x10, were A and ε resembled the absorbance and molar absorptivity,

respectively. According to the Beer's Law, for BSA under 280 nm, ε was the percent extinction coefficient (ε1%) predicted as 6.67.[9-11] Finally absorbance/time graphs were drawn.

In Vitro Chondrocyte- Biomaterial Interaction Studies

Cell culture and seeding of chondrocyte on hydrogel

Chondral tissue was harvested from the right knee of a 55-year-old female patient who underwent total knee replacement surgery. Informed consent was taken from the patient. The chondral material was treated with collagenase type II and was incubated at 37.4°C for 24 hours. After incubation, cells were centrifuged at 1200 rpm for 10 minutes and were cultured on T-25 flasks in RPMI-1640 with 10% Fetal Bovine Serum, 100 units/mL penicillin and streptomycin, insulin 5 µg, transferrin 5 µg and sodium selenoid 5 ng/ml (ITS premix solution). Complete medium was changed every other day. Human chondrocytes were also cultured throughout a period of 4 weeks according to the instructions on standard human cell cultures. Cells were included in the study after the 5th passage. Chondrocyte cultures were trypsinized and were counted by trypan blue 0.4%. They were diluted to yield 1X10^5 cells/mL in complete media. Aliquots of 20 µl of cell suspension were seeded on prewetted matrices placed in the wells of 24-well plates. Chondrocyte seeded CPBHs (1X10^5 cells/ml) were incubated for 1, 7, 14 and 21 days in the CO_2 incubator at 37.4°C. In the second group of wells, 3.24 x 10^6 numbers of cells were distributed (Figure 1). Some wells were left acellular to test the controlling release kinetics of GFs from CPBHs.

MTS assay for the proliferation of chondrocytes

Instead of Megacell RPMI-1640, DMEM was used for MTS measurements. Viability tests were performed with a commercial kit (100 ml MTS solution + 5 ml PMS solution, MTS formazan CellTiter 96 AQueous Non- Radioactive Cell Proliferation Assay) and were examined macroscopically and microscopically. Under dark room conditions, a solution of MTS was prepared with a dilution ratio of 1/6 (v/v). All samples were analyzed on day 1, 7, 14, and 21. The existing medium was removed and 500 microliters of MTS tetrazolium compound and DMEM culture medium were added to the wells. All samples were left in an incubator (%5 CO_2, 37.4°C) for 3 hours and were treated with 25 µl 10% Sodium Dodesyl Sulphate in order to stop the reaction. Afterwards, 200 microliter MTS solutions were transferred to 96-well plates and were examined at 490 nm with Elisa Microplate Reader.

Release Kinetics of CPBH

Between day 1 and 21, during the culture medium changing process at two-day intervals, 500 µl of the supernatants were placed in 1 ml Eppendorf tubes and they were read at 490 nm absorbance (OD) ELISA microplate reader. First, the amount of GFs in the culture medium was recorded so as to determine the exact amount of the GFs released from CPBH because fetal bovine serum is a component of the culture medium. To determine the exact amount of GFs released from CPBH, the amount of the GFs in a_1 (empty hydrogel) and c_1 (Empty hydrogel and chondrocyte) wells was also recorded to exclude the amount of the GFs released from the cells. Finally, daily amounts of controlled release kinetics of TGF-β1 and BMP-7 from CPBH were determined. Absorbance alterations were measured during release of GFs and absorbance/time graphs were drawn.

Statistical analysis

Canonical correlation analysis with Datafit Version 9.0.59 software package was used for statistical evaluation. The correlations of the content of the wells for each plate were calculated.

RESULTS

CHARACTERIZATION OF CPBH

SEM Analysis

The size of the pores on 2% CPBH, which has been used in experimental studies to produce human cartilage cells, were directly proportional to the degree of cross-linking in SEM images. Crosslinking was furthermore observed to be very compact (Figure 4 A, B, C, D).

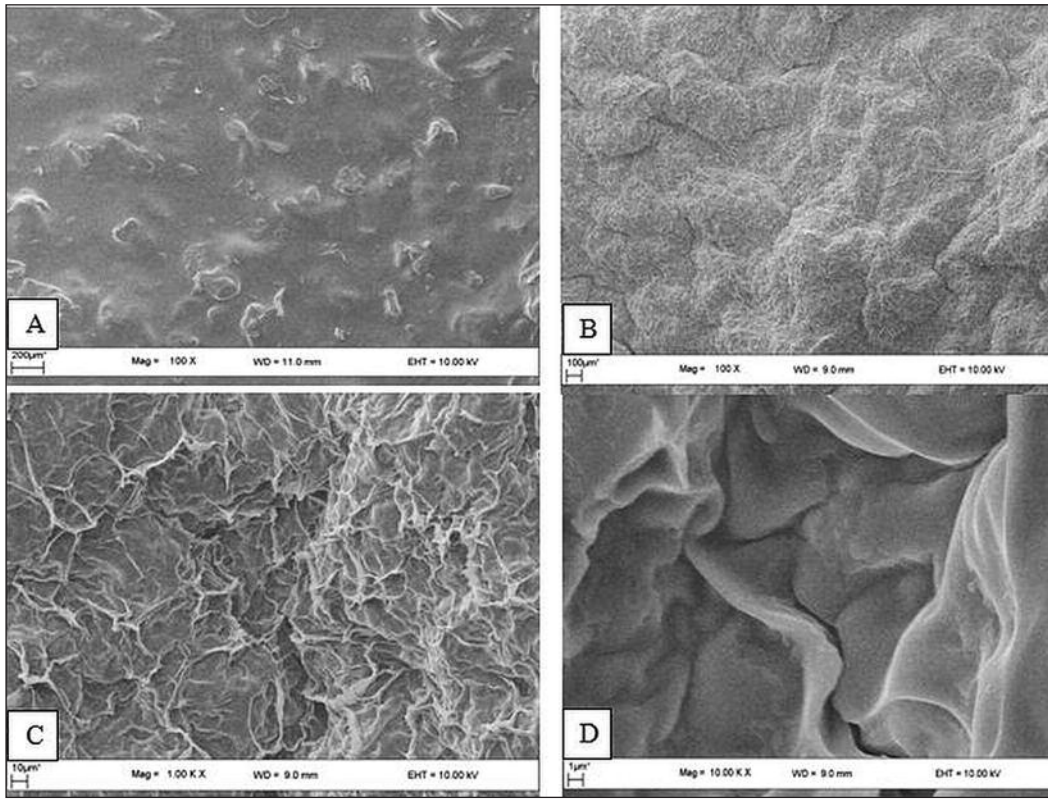

FIGURE 4: Porosity test was evaluated with scanning electron microscope analysis.

Swelling Test

Equilibrium swelling of the gels, due to the interaction between water molecules and polymer network structure, the mixture was affected by the free energy of the gel elasticity. Therefore, the environmental and synthesis conditions such as the structure of the gel network and the solvent, or the changes in the hydrophobic structure of the gel may cause changes in the gel swelling behaviour. In addition, the purity of the monomer plays an important role in the synthesis of the gel properties. Small amounts of hydrophilic, hydrophobic and ionic monomers and changes occur between the transition type and temperature during the copolymerization of the monomers.[12] Inversely affected temperature-sensitive hydrogels are made of hydrophobic polymer chains. They are used in many biomedical applications such as selective membranes, control of enzyme activities and controlled release systems. Highly balanced swelling of CPB hydrogel systems is very important for biomedical applications. Hydrophobicly modified polymeric gels with improved mechanical properties, are examples of such applications.[13,14] Many pharmacologically active compounds are used as amphiphilic or hydrophobic molecules in controlled release systems.[15,16] Amphiphilic systems can be obtained through the synthesis of polymers containing both hydrophobic and hydrophilic structures and/or weak acidic monomers.[17]

Polymers responsive to temperature and pH changes in aqueous solutions can be obtained by changing hydrophobic or hydrophilic groups in the gel structure. According to swelling tests, the behaviour of the hydrogel resembles an osmotic pump in the early period. Afterwards, the volume increases gradually, which might have corresponded to the biodegradation of the gel (Figure 5). Swelling equilibriums of the hydrogels, as shown in the chart, are the result of the interactions between the water molecules and polymer network structure.

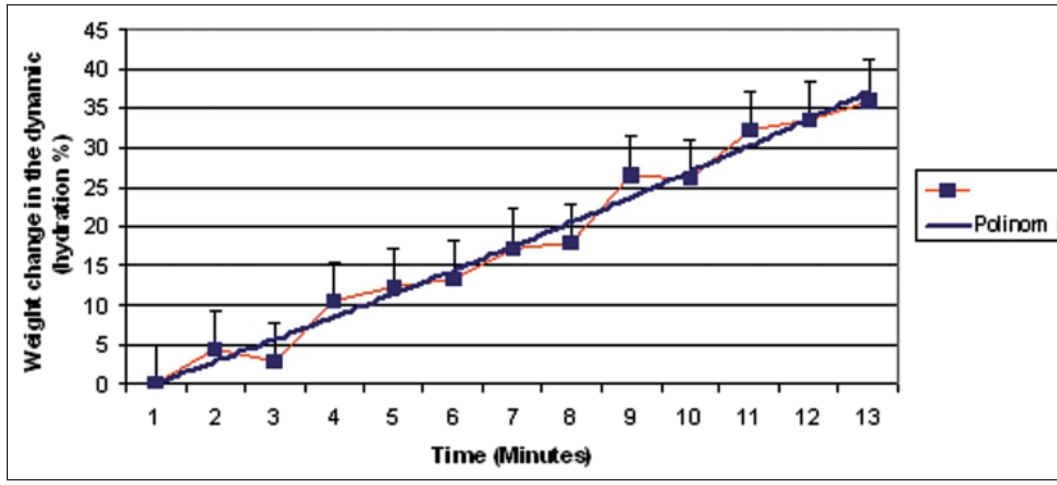

FIGURE 5: Time-depended hydration (%)-weight change of hydrogel.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

Hydrogel diameter changes depending on temperature and pH

The volume alterations of the gel in pH changes and temperature changes were recorded and average alterations were shown in graphs. Changes in the diameters of the hydrogels are directly proportional to the temperature (Figure 6). Diameters of the hydrogels decrease as the pH increases (Figure 7).

UV-spectrophotometer analysis

Absorbance alterations of empty (=blank) and BSA loaded hydrogels were performed in PBS solution with a repetition of three times and their comparative graphs were drawn (Figure 8).

Molar absorptivities of hydrogels at time intervals during release of PBS at pH 7.4 were assessed with UV spectral analysis at 280 nm wave length. Absorbance quantities and the amount of released BSA between the first and 5th days were measured, in molar concentrations of 0.2440 mg/ml and 0.6126 mg/ml, taking hydrogel without BSA as the blank. On the 6th and 7th days the values increased as a result of bio-degradation and reached to 1.3546 mg/ml molar concentration. The amount of released BSA was measured at 0.4344 mg/ml molar concentration between the first and 7th days, while the PBS was taken (pH=7.4) as the blank. These results indicated a daily regular BSA release.

Determination of Chondrocyte Growth on CPBH

The total count of living cells seeded on hydrogels in c wells was assessed with MTS.

Actual number of alive cells was determined with the ELISA plate reader, by substracting absorbance values of chondrocyte lacking groups from chondrocyte containing groups.[8,14,18] During the whole experiment process, the viability rates in c_4 wells, in which the GFs were released consecutively, was much higher than the others (3.24 x

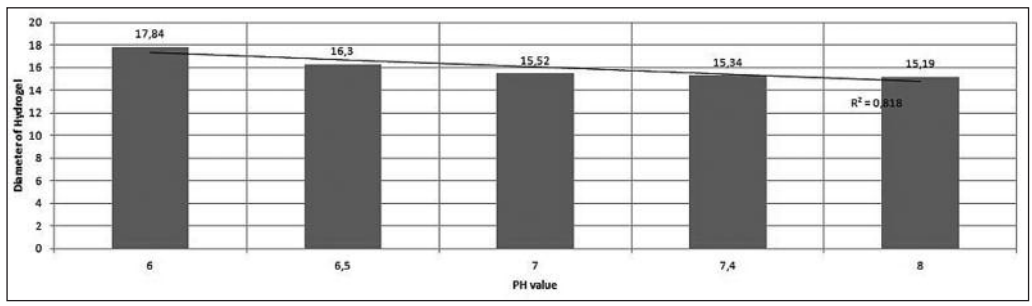

FIGURE 6: Diameters of the hydrogels in response to pH.

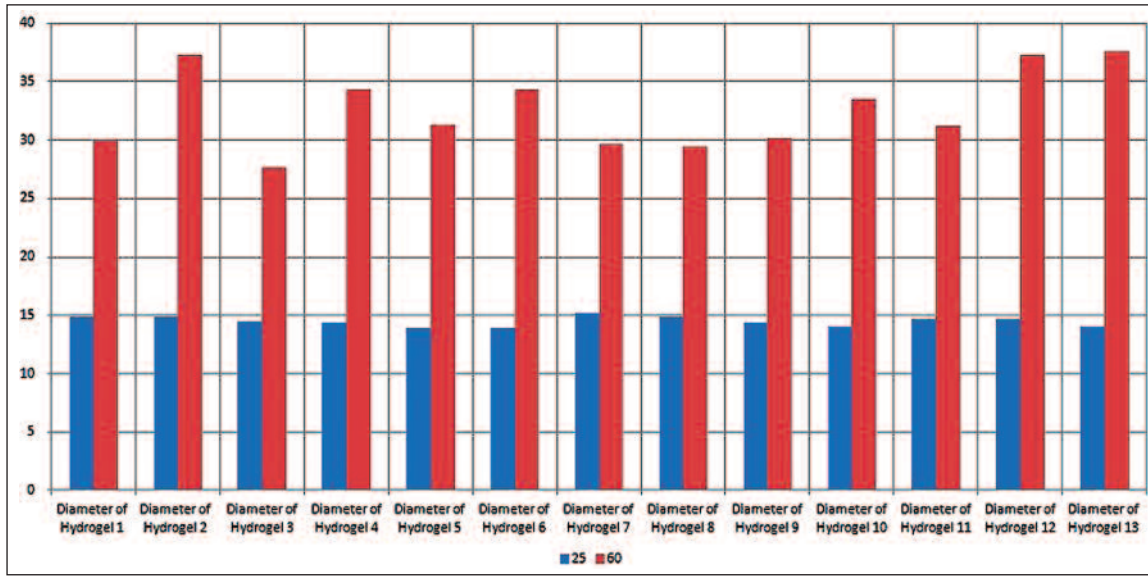

FIGURE 7: Diameters of the hydrogels in response to temperature.
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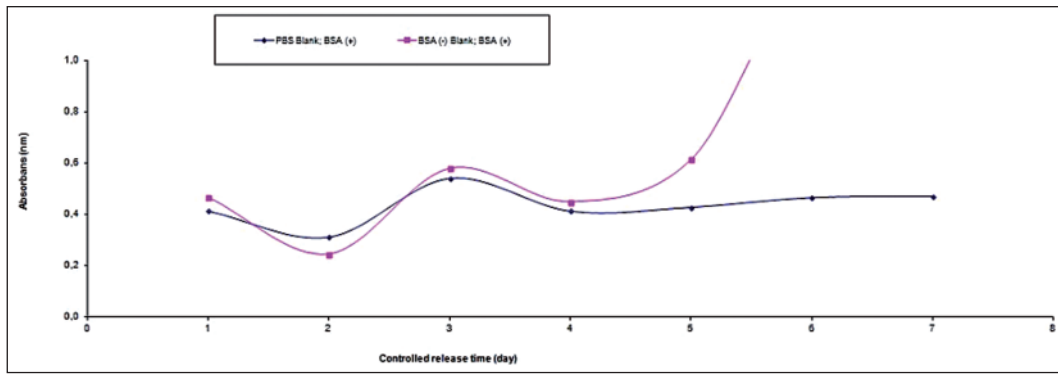

FIGURE 8: Absorbance change against time during release of the hydrogels in pH=7.4 phosphate buffer.
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10^6). Significant difference was observed with combined signalling with both the TGF-β1 and BMP-7 groups (Figure 9-11).

The increase in the amount of the chondrocytes showed that CPBH had no toxicity on cells.

Evaluation of Kinetics of Release by ELISA

Release Kinetics of TGF-β1

Non-linear formulas, which might help to calculate the release amount of TGF-β1 within the wells, were presented in Table 1. As already mentioned, since c_3 wells contained only BMP-7, TGF-β1 assessment from this well was incongruous (Table 1).

The amount of the release of TGF-β1 from CPBH was given in Table 2.

The hydrogel system including combined GFs seemed to allow TGF-β1 release after day 9, after the amount of the TGF-β1 in the culture media (0.056%) and the amount of the TGF-β1 produced by the chondrocytes (0.92%) were excluded.

At the end of day 21, 98.34% of TGF-B1 was released (Figure 12).

The remaining amounts of GFs were considered to be absorbed by the cells or CPBH itself.

Release Kinetics BMP-7

Non-linear formulas, which might help to calculate the release amount of BMP-7 within the wells, were presented in Table 3. As already mentioned, since c_2 wells contained only TGF-B1, BMP-7 as-

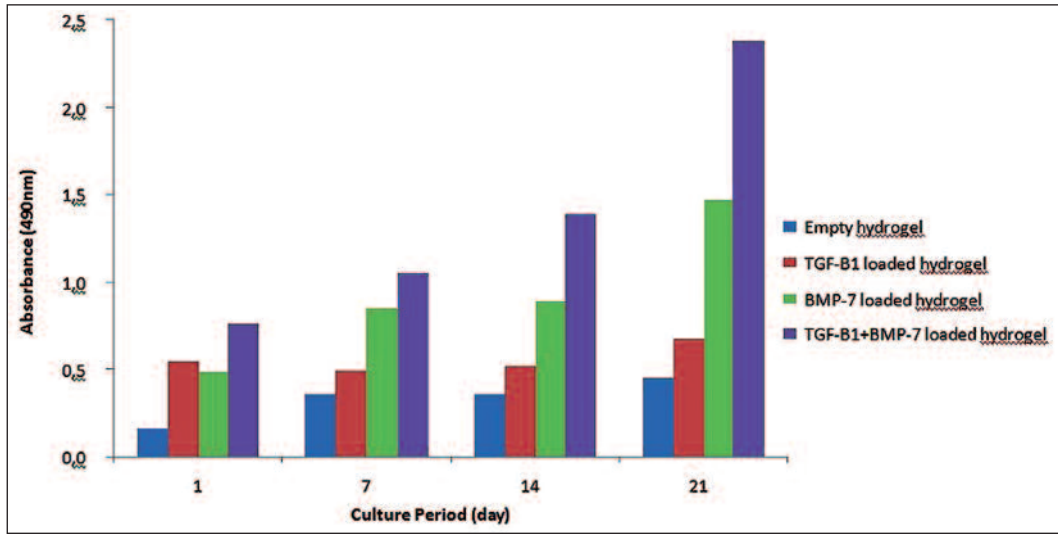

FIGURE 9: CellTiter 96® Non-Radioactive Cell Proliferation (MTS) Cell Proliferation enzyme-linked immuno assay.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

sessment from this well was incongruous (Table 3).

The amount of the release of BMP-7 from CPBH in different wells was given in Table 4.

The hydrogel system that included combined GFs seemed to allow BMP-7 release during the initial 9 days, after the amount of the BMP-7 in the culture media (0.19%) and the amount of the BMP-7 produced by the chondrocytes (0.82%) were excluded.

At the end of day 21, 82.62% of BMP-7 was released (Figure 13). The remaining amounts of GFs were considered to be absorbed by the cells or CPBH itself. During the first 9 days BMP-7 and afterwards until day 21, TGF-β1 were therapeutically and separately released from the c_4 well.

Statistical Analysis

Regression equations were shown in Tables 1 and 3 (For TGF-B1, and BMP-7).

DISCUSSION

In order to prevent a sudden release of drugs in the body for the development of appropriate delivery systems, it is very important to use polymers which have low toxic effects and which are biodegradable.[19] In this context, well-known pharmaceutical-chemical and biological properties of chitin and chitosan derivatives and nano-microspheres, nano-microcapsules and beads created after polymerization, ionotropic gelation, etc. have great potential as drug delivery systems. The hydrogel, whose effectiveness is the most well-known among the hydrogels, has been conveniently used for the controlled release of high amounts of drugs as well as a drug delivery system.[20,21] There are several investigations enhancing existing applications of the ideal controlled release formulations and utilization of polymers such as chitin and chitosan derivates.[22] Chitosan is non-toxic, biodegrades without harming the environment, degrades to completely harmless products (amino sugar) within the body and has no side effects.[23] These properties give it great importance in terms of its use in cell and tissue culture studies in pharmaceutical fields.[24,25]

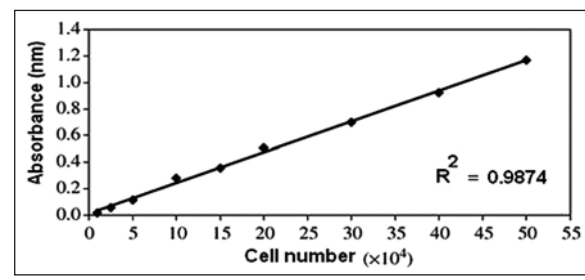

FIGURE 10: CellTiter 96® Non-Radioactive Cell Proliferation (MTS) calibration curve for chondrocyte determination (OD_{490}).

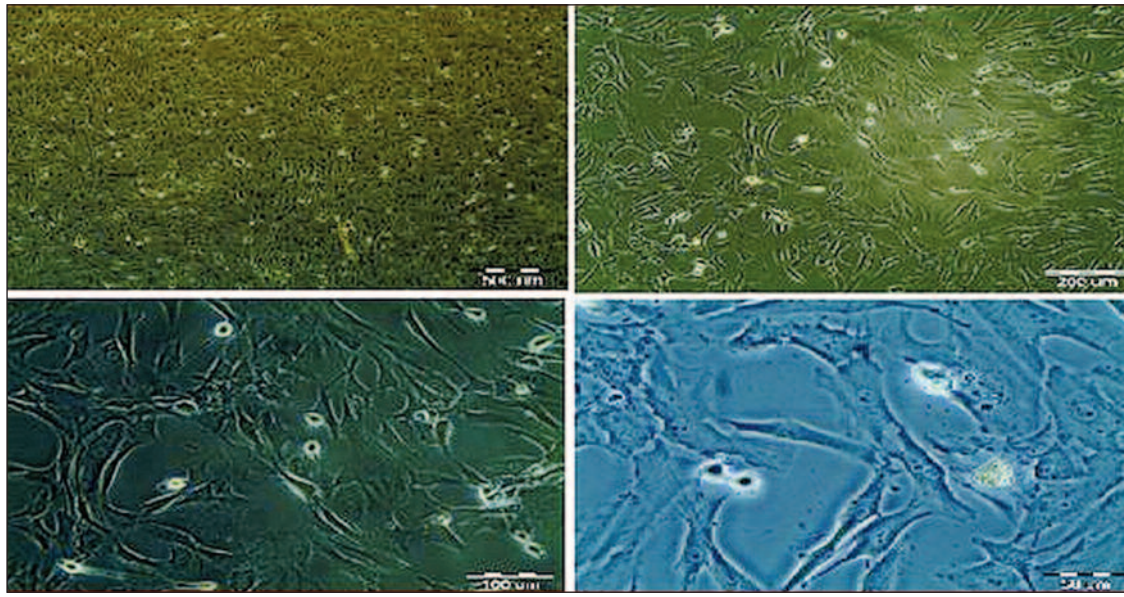

FIGURE 11: Chondrocytes seeded to chitosan-poly-borax (CPB) hydrogel (X4, X10, X20, X40 magnified) on inverted microscope at day 14.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

TABLE 1: Regression equations and determination coefficients (R^2) for a_3, a_4, c_2, c_3 ve c_4 wells were given. The correlation coefficients for a_3, a_4 and c_4 wells were significant ($p<0.001$). Correlation coefficient in c_2 was also significant ($p<0.05$). Correlation in c_3 well was insignificant [transforming growth factor (TGF)-β1-free hydrogel class].

	Equation	Model Definition	R^2	p
a_3	Y = a+b*x^3+c*exp(x)	Absorbance (nm) = $4.65*10^{-2}$ + $4.01 * 10^{-5} * day^3$ -$4.29 *10^{-10} *$ exp(day)	0.808	0.001**
a_4	Y = a+b*x^3+c*exp(x)	Absorbance (nm) = $2.22 * 10^{-2}$ + $4.36 * 10^{-5} * day^3$ -$5.09 * 10^{-10}$*exp(day)	0.915	0.001**
c_2	Y = a+b*x^3+c*exp(x)	Absorbance (nm) = $4.84 * 10^{-2}$ + $2.59 * 10^{-5} * day^3$ -$3.28 * 10^{-10} *$ exp(day)	0.483	0.099
c_3				
c_4	Y = a+b*x^3+c*exp(x)	Absorbance (nm) = $1.98 * 10^{-2}$ + $5.71 * 10^{-5} * day^3$ - $7.61 * 10^{-10} *$ exp(day)	0.813	0.001**

**Significant.

Chitosan is biocompatible with organic compounds; it forms gels and precipitates after crosslinking with multivalent anions It is an ideal biomaterial to create a scaffold.[26] Highly deacetilated chitosan has a high mitogenic activity.[27,28] Chitosan is also a demanding biomaterial in organogenesis. Chitosan is soluble in diluted acids such as hydrochloric acid, acetic acid and formic acid. It becomes water-soluble as a result of the protonization of its amino groups.[29,30]

Chitin and chitosan derivatives with these characteristics were used as biomaterials and in tissue engineering in a wide range.[31-33] In medicine, they are applicable as surgical materials, artificial skin and organs.[34] In the present study, to the best of our knowledge, we report a first example of hydrogel, consisting of CPB, which may promote cartilage repair as the native hyaline tissue.

PVA is highly hydrophilic, nontoxic and biocompatible with excellent film forming property. PVA films have good mechanical strength, and long-term thermal and pH stability. These characteristics of PVA allow their use in areas of medical and pharmaceutical applications. Cross linked PVA membranes show good swelling property and are useful in sustaining drug release.

The deacetylation degree of chitosan was shown to increase cell adhesion and proliferation.[35] This was our reason to use 91% deacetylation degree of chitosan in order to increase the adhesion and proliferation of the cartilaginous cells.

Borax, which is a cross-linker suitable for polymer containing hydroxyl groups, has been used in the field of pharmaceutics.[36] In the present study, to the best of our knowledge, we report a very first example of drug delivery system consisting of chitosan, PVA and borax. Chitosan and PVA forms weak ionic links, and these links are enhanced by the crosslinker, borax, so as to increase the time for biodegradation. Borax makes our hydrogel completely different and special from those presented in the literature, leading to stronger links between PVA and chitosan.

Polymer systems like chitosan based hydrogels have well-known pharmacokinetic mechanisms that control the delivery of the carried GF. Chitosan (=deacylated Chitin) was preferred as the main component of a hydrogel to form a biocompatible, adhesive, surgically applicable, biodegradable, elastic, volumetrically stable, transfer capable biomaterial with a high amount of small sized porous matrices.[37,38] Furthermore, the binding capability of the chondrocytes to the surface of the chitosan polymer has been shown by the transmission electron microscope.[39] The velocity of the release from the polymer was controlled in diffusion mechanisms by adjusting the pore diameters towards shrinking with crosslinkers. Borax was our choice as the crosslinking agent to strengthen the ionic crosslinks. Anabolic and stimulating effects of GFs are well known issues in the literature.[40,41] Both GFs in our study were previously used by other investigators owing to their different anabolic features.

TABLE 2: Transforming growth factor (TGF)-β1 release in a_2, a_4, c_2, c_3, c_4 wells, at 2-day intervals for 21 days. TGF-β1 release in c_3 well at 2-day intervals for 21 days was insignificant.

Days	a_2 well	a_4 well	c_2 well	c_3 well	c_4 well
1	0.76%	0.2%	1.16%		0.16%
3	1.98%	0.32%	1.96%		0.52%
5	2.44%	0.38%	3.34%		0.82%
7	2.62%	0.42%	3.88%		0.96%
9	3.8%	1.08%	4.06%		1.28%
11	3.22%	1.56%	4.68%		1.44%
13	3.38%	1.56%	4.02%		1.86%
15	3.42%	4.14%	4.98%		4.1%
17	3.62%	4.74%	4.28%		4.34%
19	5.58%	4.14%	5.18%		5.86%
21	3.18%	2.64%	2.12%		2.64%

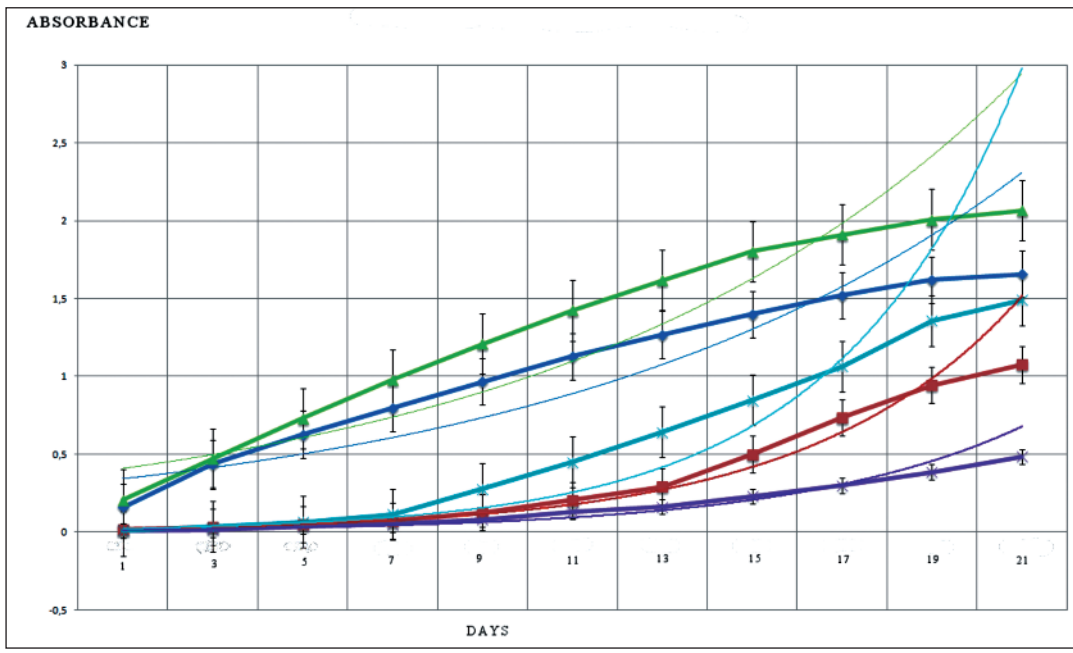

FIGURE 12: Controlled release kinetics of transforming growth factor (TGF)-β1 from chitosan-poly-borax (CPB) hydrogel. Sustained release of TGF-β1 in culture media at 37.4°C was observed over 21 days.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

TABLE 3: Regression equations and determination coefficients (R^2) for a_3, a_4, c_2, c_3 ve c_4 wells were given. Correlation coefficients for a_3, a_4, and c_4 wells were significant ($p<0.001$). Correlation coefficient in c_2 was insignificant [bone morphogenic protein (BMP)-7-free hydrogel class].

	Equation	Model Definition	R^2	P
a_3	y = a*x + b	Absorbance (nm) = -0.1471 * day + 2.9492	0.908	0.001**
a_4	y = a*x + b	Absorbance (nm) = -0.1102 * day + 2.6147	0.835	0.001**
c_2				
c_3	y = a*x + b	Absorbance (nm) = -0.1153 * day + 2.7363	0.825	0.001**
c_4	y = -a*x + b	Absorbance (nm) = -0.1063 * day + 2.7494	0.880	0.001**

**Significant.

Hydrogels, which are able to respond to the environment, are the most important polymers responding to pH or temperature, or both.[42] Hydrogels with these properties have been reported to be appropriate for protein releases.[43,44]

The hydrogel used in this study was temperature and pH dependent, and may be mentioned as a carrier ideal for protein structured growth factors (Figures 6, 7).

Hydrogels behave according to the pH, temperature and ionic strength of the physiological medium.[45-48] Properties of hydrogels depend on their anionic, cationic, or neutral structures. The ionic interaction between the charged polymers and the free ions play an important role in the swelling of ionic hydrogels. Ionic hydrogels swell more than the neutral hydrogels. In this case, drug or protein transfer in ionic gels are significantly different from neutral gels.[49] Biological activity is maintained until the completion of drug release from hydrogels.[50] Release from hydrogels depends on the degree of swelling of the hydrogel and swelling depends on the degree of crosslinking. Hydrogels which contain more crosslinkers are more compact and have restricted chain movements leading to decreased swelling and vice versa. Therefore, by adjusting the percentage of crosslinking degree of desired swelling, elastic hydrogels can be prepared in accordance with the surgical technique.

In this study, we prepared a drug delivery system of desired mechanical properties, consisting of CPB hydrogel. Its swelling degree could be adjustable and crosslinking percentage was increased with 18% borax.

TABLE 4: Bone morphogenic protein (BMP)-7 release in a_3, a_4, c_2, c_3, c_4 wells at 2-day intervals for 21 days. BMP-7 release in the c_2 well at 2-day intervals for 21 days was insignificant.

Days	a_3 well	a_4 well	c_2 well	c_3 well	c_4 well
1	10,5%	9.18%		12.39%	13.34%
3	14.04%	10.38%		8.23%	8.37%
5	10.4%	11.57%		12.14%	10.03%
7	7.1%	7.48%		5.73%	9.57%
9	7.85 %	7.68%		8.24%	6.65%
11	4.87%	4.2%		7.20%	6.69%
13	3.96%	7.31%		6.29%	7.36%
15	2.36%	3.78%		4.17 %	5.20%
17	2.37%	3.60%		3.52%	4.12%
19	0.98%	1.98 %		2.53%	3.38%
21	0.69%	1.34%		1.22%	2.30%

Unreacted remnant monomers, oligomers or crosslinkers were reported to cause toxic effects.[51] The relationship between the chemical structures and toxicities of acrylate and methacrylate monomers was examined and crosslinking with gamma irradiation was reported to be the best solution to the problem of toxicity of the hydrogels.[52-54] In this study, borax was used as the crosslinking agent, and no toxicity was demonstrated (Figures 9-11).

Growth factors play an important role in the regeneration of cartilage.[55] However, these growth factors need to be released in a controlled manner in effective dose and durations to the desired regions. TGF-^{B}1 may cause fibrosis and hypertrophy when released in an uncontrolled and long-lasting manner, and it also may lead to the formation of osteophytes in cartilaginous regions.[5,6] Therefore,

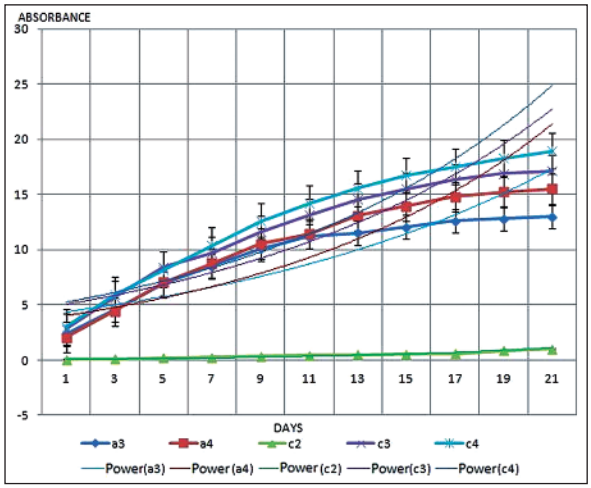

FIGURE 13: Controlled release kinetics of bone morphogenic protein (BMP)-7 from chitosan-poly-borax (CPB) hydrogel. Sustained release of BMP-7 in culture media at 37.4°C was observed over 21 days.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

it is crucial to apply the delivery systems and TGF-β1 to the cartilage in a controlled manner and in optimum conditions. For this purpose, we have designed CPB hydrogel from which BMP-7 is released first and rapidly and TGF-β1 consecutively and slowly, in order to form hyaline cartilage instead of fibrous cartilage in the desired region.

In-vitro release kinetics of CPB hydrogels were analyzed by UV-spectrophotometer and ELISA, in this study. As a result, in c_4 wells, in which BMP-7 and TGF-β1 were combined and were consecutively released, 82.62% of BMP-7 and 98.34% of TGF-B1 were seen to be released at the end of day 21. The correlations of the content of wells for each plate were calculated with canonical correlation analysis. However, it must be identified and explained in detail that diffusion and release mechanism has full compliance according to the Fick's law. If any deviation is shown, it must be evaluated with the case II transport mechanism.[56-58] Further study might be conducted in order to precisely determine the release kinetics of our CPBH. Zero and first-order kinetics and their goodness of fit analysis according to Higuchi and Hixon Crowell equivalents may be an issue for another experimental study.

CONCLUSION

From the clinical perspective, as an outcome from this study, readers may be encouraged to use the novel hydrogel in humans as an ideal scaffold. Studies about the biomechanic features of the aforementioned polymer are under investigation. The present study is a preliminary report suggesting that the hydrogel may be suitable for the chondrocites. Consecutively controlled release of the GHs from this hydrogel, may be a new horizon on chondrocyte regeneration studies.

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