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# PROLIFERATION OF MC3T3-E1 OSTEOBLASTIC CELLS AFTER SEQUENTIAL RELEASE OF PDGF AND BMP-6. AN IN VITRO STUDY

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## ABSTRACT

**Background and Aim:** To evaluate the release kinetics of bone morphogenetic protein (BMP)-6 or -7 loaded nanoparticles (NPs) that located inside the microparticles (MPs) carrying platelet-derived growth factor (PDGF), and test this NP-in-MP system with MC3T3-E1 osteoblastic cells.

**Materials and Methods:** Poly-lactic acid-co-glycolic acid (PLGA) NPs was loaded with BMP-6 or -7 and inserted in sodium alginate MPs loaded with PDGF. To evaluate the osteoblastic effect; proliferation of MC3T3-E1 cells that were treated with BMP-6, -7 and PDGF free solutions (FS) or within the particles (BMP-6 or -7 loaded PLGA NP alone and BMP-6 or -7 loaded PLGA NP in PDGF loaded alginate MP) were assessed at 2, 4, 7, 14 and 21 days.

**Results:** It was shown that while both NP and NP-in-MP systems showed similar burst release at first time periods; especially in 24-72 h time period, NP-in-MP system exhibited a sustained release profile till 14th day. According to proliferation experiments, till the 7th day, both particle and FS groups exhibited similar profiles, but after that time particle groups, especially BMP-7 NP in PDGF MP, reached to statistically higher cell numbers than FS groups. NP-in-MP system exhibited a gradually longer time factor release resulting with delayed but elongated cell proliferation period.

**Conclusion:** Findings indicate that NP-in-MP system might be promising in future for mimicking the natural bone formation process by providing sequential release of PDGF and BMPs, for bone tissue engineering. More comprehensive experiments evaluating mineralization and gene expression profile is necessary to verify these results.

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**Keywords:** Alginate Microparticles, Bone Morphogenetic Protein-6, Bone Morphogenetic Protein-7, Periodontal Tissue Engineering, PLGA Nanoparticles, Sequential Release

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# PDGF VE BMP-6'NIN ARDIŞIK SALIMI SONRASI MC3T3-E1 OSTEOBLAST HÜCRELERİNİN PROLİFERASYONU. IN VITRO BİR ÇALIŞMA

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## ÖZ

**Amaç:** Mevcut çalışma trombosit kaynaklı büyüme faktörü (platelet-derived growth factor, PDGF) taşıyan mikropartiküller (MP) içerisine hapsedilmiş nanopartiküller (NP)'den kemik morfogenetik protein (bone morphogenetic protein, BMP)-6 veya -7'nin salım kinetiğini değerlendirmek ve bu sistemi MC3T3-E1 osteoblastik hücreleri ile test etmektir.

**Gereç ve Yöntemler:** Polilaktik asit-glikolik asit (PLGA) NP'ler BMP-6 veya BMP-7 ile yüklenmiş ve PDGF ile doldurulmuş sodyum aljinat MP'lere yerleştirilmiştir. Osteoblastik etkiyi değerlendirmek için serbest veya partiküller içerisinde yüklü (BMP-6 veya BMP-7 yüklenmiş PLGA NP ile PDGF yüklenmiş aljinat MP içerisinde BMP-6 veya BMP-7 yüklenmiş PLGA NP) BMP-6, -7 ve PDGF ile muamele edilip 2, 4, 7, 14 ve 21. günlerde incelenmiştir.

**Bulgular:** İlk zaman periyotlarında hem NP hem de MP içerisinde NP sistemleri benzer patlayıcı salım miktarı gösterirken özellikle 24-72 saat zaman diliminde MP içerisinde NP sistemi 14. güne kadar uzamış bir salım profili ortaya koymuştur. Proliferasyon deneylerine göre 7. Güne kadar her iki partikül grubu ve serbest solüsyon grupları benzer profiller oluşturmuştur ancak bu periyottan sonra özellikle PDGF MP içerisinde BMP-7 NP grubu olmak üzere partikül grupları, FS gruplarına kıyasla istatistiksel olarak anlamlı düzeyde daha fazla hücre sayısına ulaşmıştır. MP içinde NP sistemi ise dereceli ve daha uzun süren bir faktör salımı yaparak gecikmiş ve uzamış bir yüksek hücre proliferasyon periyodu sağlamıştır.

**Sonuç:** Mevcut deneylerin bulguları MP içinde NP sisteminin PDGF and BMP'lerin ardışık salımını sağlayarak gelecekte kemik doku mühendisliğinde doğal kemik formasyon sürecini taklit edebileceği konusunda ümit vermektedir. Ancak bu sonuçları doğrulamak için mineralizasyon ve gen ekspresyon profillerini içeren daha kapsamlı deneylere ihtiyaç vardır.

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**Anahtar Kelimeler:** Aljinat Mikropartiküller, Kemik Morphogenetik Protein-6, Kemik Morphogenetik Protein-7, Periodontal Doku Mühendisliği, PLGA Nanopartiküller, Ardışık Salım

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## INTRODUCTION

Regeneration of the bony defects that occur due to chronic diseases or mechanical trauma is still a challenging medical issue. Although many approaches including autogenous bone, allografts and alloplasts have been used, an ideal material for reconstruction of such defects still does not exist. Today, the way of research seems to be directed to tissue engineering based procedures. In vitro and in vivo studies show promising results in providing a basis to apply such therapeutic strategies for bone defect repair.

When the properties of bone biology were reviewed, the influence of bioactive molecules that circulate within the fluids and reside in the bone matrices is, a well-known issue in bone healing, regeneration and remodeling.[1] Of these molecules, bone morphogenetic proteins (BMPs), a member of transforming growth factor-beta (TGF-β) family and within their types, around 20, only five (-2, -4, -6, -7 and -9) have displayed remarkable osteogenic activities.[2-4] Many cell types assigned in fracture healing have expressed BMPs suggesting their regulatory role in bone repair.[5] Among BMPs, -2 and -7 are the most extensively studied molecules and, their clinical use has been approved. BMP-6 is the relatively less investigated bioactive molecule and potential of its biologic activities is not yet fully defined. The enhancing capacity of BMP-6 in alveolar bone formation[6], role in mesenchymal stem cell regulation[7] and osteogenic differentiation[8] has been previously reported. Suppression of the BMP-6 signaling pathway results in accumulation of the MSCs in the bone marrow without differentiation due to specific signal deficiency.[9-11] It has been shown that exogenous BMP-6 is more potent in vitro compared to BMP-2 and BMP-7 with regard to induction of bone nodule formation and MSCs differentiation towards osteoblasts. It was also reported that while BMP-6 reduced the number of osteoclasts derived from hematopoietic stem cells, BMP-2 and BMP-7 increased their numbers.[12]

The osteoinductive properties of BMPs might be combined with other growth factors (GFs) such as platelet-derived growth factor (PDGF), TGF, and insulin-like growth factor (IGF) etc. Among these GFs, PDGF is thought to be one of the key regulators in general tissue repair.[13] After an injury, blood platelets release PDGF and wound healing process is enhanced by chemotaxis and proliferation of cells.[14,15] With the influence of PDGF, various bone cell types and their surrounding tissues also show increased proliferation and angiogenesis.[16,17] Although angiogenesis can be stimulated by both PDGF and VEGF, PDGF uniquely stimulates the mesenchymal cell migration.[16,17] Its therapeutic potential in skeletal reconstruction was also demonstrated using different animal models.[13] In terms of bone healing and regeneration, PDGF is released at early periods of cell response and responsible from cell proliferation whereas BMPs mainly take role in osteoblastic cell differentiation that occurs at later stages of bone formation. Therefore, enhancing the concentrations of these bioactive mediators with ideal timing by using a sequential release system might be considered as a strategy.

According to the common findings of abovementioned studies, prevention of the burst release of such vehicles and thus, providing their sustained release and activity in the healing area is still an obstacle for tissue engineering applications.[18] Starting from this point, several attempts including preparation of controlled release systems involving 3D scaffolds and nano/microparticles.[19] Despite several studies evaluating the in vitro effects of particles on bone forming cells, into our knowledge, there is still lack of knowledge about a system embedding nanoparticles (NPs) into microparticles (MPs) and providing sequential release of PDGF and BMPs. Therefore, the aim of this study is to evaluate the release kinetics of BMP-6 loaded nanoparticles that reside in the MPs carrying PDGF and test this NP-in-MP system in vitro using osteoblastic cells. With the present system, it was aimed to provide a sequential release system constituting a early PDGF release and followed by delayed BMP-6 release system mimicking the biological process of natural bone formation.

## MATERIALS AND METHODS

### *Nanoparticle preparation*

Water-in-oil-in-water (w/o/w) double-emulsion method was used to prepare the NPs. The concentration of either BMP-6 or BMP-7 was 10 µg/ml and dissolved in aqueous media that contains Pluronic F68 as surfactant. Meanwhile, PLGA was dissolved in dicloromethane at 100-mg/ml concentrations and mixed with the above-mentioned solution. A probe sonicator was used during 3 min to build a water-in-oil emulsion. Then, the emulsion was added into 50 mL of aqueous solution to obtain (w/o/w) double emulsion system and subjected to magnetic stirring at 300 rpm for 4 hours.

Then, NPs were collected after centrifugation, washing and lyophilizing.

### *Nanoparticle characterization*

Scanning electron microscopy was used to examine the morphology of the NPs (Evo 50, Zeiss, Oberkochen, Germany). NPs were re-suspended after centrifugation and washing with deionized water. To obtain the desired images, one drop was put on a glass and waited for drying then coated with Au-Pd. Images were taken at 15000 X magnification with 15 keV acceleration voltage.[20] Zetasizer Nano ZS90 (Malvern Instrument, UK) device was used to evaluate the sizes and zeta potentials of the NPs.[21] The measurements were performed as triplicate. To clearly distinguish their surface morphology, NPs were coated by using gold palladium layer and displayed under scanning electron microscope (SEM) (Zeiss Evo 50, Oberkochen, Germany).

### *Incorporation of nanoparticles into microparticles*

NPs were incorporated into MPs by the method previously used by Garrait et al.[22] An experimental setup consisting of a syringe pump, syringe and a beaker containing $CaCl_2$ solution as the cross-linker was used to obtain alginate MPs. In a typical batch, alginate (high molecular weighted, viscosity 1000 cps) was dissolved in phosphate buffer saline (PBS), containing 1 µg/mL PDGF, at a concentration of 2% (w/v). The solution was added drop wise into the $CaCl_2$ solution (2% w/v). In order to obtain sequential release system (NP-in-MP) 2% sodium alginate solution containing 1 µg/mL PDGF was combined with NPs with predetermined BMP-6 or BMP-7 concentrations (100 ng per batch). Then, the droplets were collected and freeze dried.

### *Characterization of microparticles*

Morphologies of the MPs were examined under inverted optical microscope (Nikon Corporation, Japan). The images were obtained under 4x magnification and Image J program (NIH, Bethesda, Maryland, USA) was used to measure approximately a hundred particles.

### *Degradation study*

Lysozyme was used in testing the in-vitro degradation of MPs. First, MPs were kept in PBS (pH 5.1 and 7.4) for 30 weeks. Then, the enzyme (10 mg/L lysozyme) was added into the PBS and incubated under 37°C with continuous shaking (50 rpm). The mean change in the solution pH for 5.1 and 7.4 was detected during 30 weeks period.[23]

### *In vitro release studies*

For testing the in vitro release system, only particles loaded with BMP-6 were used to determine release kinetics. The amount of released BMP-6 at certain time intervals in NP-in-MPs and NPs was detected by re-suspending the precipitated particles (1 mg) in PBS (1 mL, pH=7.4) and incubating under light agitation (37°C). At 1st, 2nd, 24th hours and 3rd, 7th, 14th and 21st days, liquid samples were collected and frozen at −20°C until analysis. After defrosting, the samples were centrifuged and in 1% (v/v) acetic acid at 37°C for 24-48 h. Then, the amount of BMP-6 was measured by using an ELISA kit.[23]

### *Preparation of MC3T3-E1 cells*

MC3T3-E1 cells were recruited from mouse osteogenic cell line (Subclone 4 CRL-2593™, ATCC® Cell Bank, London, UK). After their dissolving process, subculture of the cells was carried out in flasks containing Minimum Essential Medium (α-MEM) (Sigma Co.) + fetal bovine serum (10%) (v/v) (FBS, Sigma Co.) + Penicillin-Streptomycin (1%). Maintaining of the cells was carried out at 37 °C atmosphere humidified with $CO_2$ (5%). Before cell seeding, dissociation of the cells was made by following the steps of trypsin-EDTA (0.25%) (Sigma Co.) addition, centrifugation and resuspension with medium.

### *MC3T3-E1 cell culture experiments*

The cells were cultured in sterile, stabilized 48-well TCPS dishes. Before the experiments, sterilization of the dishes were provided by coating with parafilm, being soaked in ethanol (96%) and positioning under ultraviolet light during 30 min. Chitosan alginate NPs and MPs were sterilized with 25 kGy gamma irradiation[24] and their equilibration was done by placing inside sterile Dulbecco's PBS (pH=7.4) for 24 h. Before cell seeding, conditioning of the particles were carried out with α-MEM including FBS (10%) for 1 h. 50 mL of the cell suspensions were also injected into each group samples and incubated during 1h at the environment with 37°C and $CO_2$ (5%). Then, the cell culture residing in α-MEM+PBS (10%) + ascorbic acid (50 mg/mL) + b-glycerol phosphate (10 mM) was added to each well by considering the inoculation density of $4x10^4$ cells/mL for each group. The medium was changed with fresh one at every two days.

Cell culture experiments were consisted of following groups: BMP-6 FS (100 ng/well), BMP-7 FS (100 ng/well), PDGF FS (100 ng/well), PDGF loaded alginate MP (20 ng protein per well), BMP-6 loaded PLGA NP (100 ng protein per well), BMP-7 loaded PLGA NP (100 ng protein per well), BMP-6 loaded PLGA NP in PDGF loaded alginate MP, BMP-7 loaded PLGA NP in PDGF loaded alginate MP (and control group (α-MEM + PBS (10%) + ascorbic acid (50 mg/mL) + b-glycerol phosphate (10 mM). The mixtures residing the GF solutions or particles were directly added to their related wells. Proliferation of the MC3T3-E1 cells were evaluated at days 2, 4, 7, 14 and 21 using cell counting kit WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] in triplicate. For determining the proliferation, 10 mL WST-8 solution was added to culture well and an incubation period of 2.5 hours was given. Microplate reader was used to measure the amount of yellow colored product and the amount was calculated by considering as directly proportional to the viable cell count.[25]

### *Statistical analysis*

Data are expressed as median (min-max) and mean ± SD of a representative of each sample group (n=3) on each cultivation day. Statistical evaluations were made by Kruskal Wallis (inter-group differences) and Friedman (time-dependent changes) tests. Dunn test was used for post-hoc pair-wise comparisons of the differences. Statistical analysis was performed using IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY, USA) software.

## RESULTS

### *Characterization of nanoparticles*

Both empty and loaded NPs were positively charged. Since BMPs were negatively charged, their addition into NPs decreased the zeta potential values. On the other hand, hydrodynamic diameter of empty NPs was calculated as 296 nm by zetasize data and particle size remained stable with the increasing BMP-6 concentrations (Table 1). Since the concentration of BMP-6 was within its biologic activity range (0.1 to 10 ng/mL), the loading was considered as acceptable. SEM images of the NPs are shown in Figure 1. Although aggregates were seen in the sample, surface morphology of NPs was rough and they exhibited well dispersed nature in aqueous media.

### *Characterization of microparticles*

MP obtained with one needle did not present significant size variability at repetitive synthesis. Inverted light microscope views of the MPs showed almost spherical forms and the diameters of MPs were between 50 and 80 μm (Figure 2).

### *Degradation of microparticles*

To evaluate the structural stability of MPs, their in vitro degradation was evaluated. During 30 weeks, the pH values of the solution containing the MPs did not show

**Table 1.** Zeta potential values of the microparticles

| | BMP-6 (μg /ml) | Particle size (nm) | | Zeta potential (mV) | |
|---|---|---|---|---|---|
| | | Mean±SD | Median (min-max) | Mean±SD | Median (min-max) |
| I | 0 | 296.0 ± 6 | 296.0 (290.0-302.0)[a] | 44.4 ± 0.9 | 44.4 (43.5-45.3)[b] |
| II | 1 | 273.5 ± 5 | 273.5 (268.5-278.5) | 36.5 ± 0.8 | 36.5 (35.7-37.3) |
| III | 1.5 | 265.0 ± 7 | 265.0 (258.0-272.0) | 34.7 ± 0.6 | 34.7 (34.1-35.3) |
| IV | 2 | 287.0 ± 6 | 287.0 (281.0-293.0) | 32.9 ± 0.7 | 32.9 (32.2-33.6) |
| V | 2.5 | 274.0 ± 8 | 274.0 (266.0-282.0) | 31.7 ± 0.8 | 31.7 (30.9-32.5) |
| VI | 3 | 269.5 ± 9 | 269.5 (260.5-278.5) | 30.9 ± 0.7 | 30.9 (30.2-31.6) |

BMP, bone morphogenetic protein; a, significantly higher than 1.5 μg/ml; b, significantly higher than 3 μg/ml

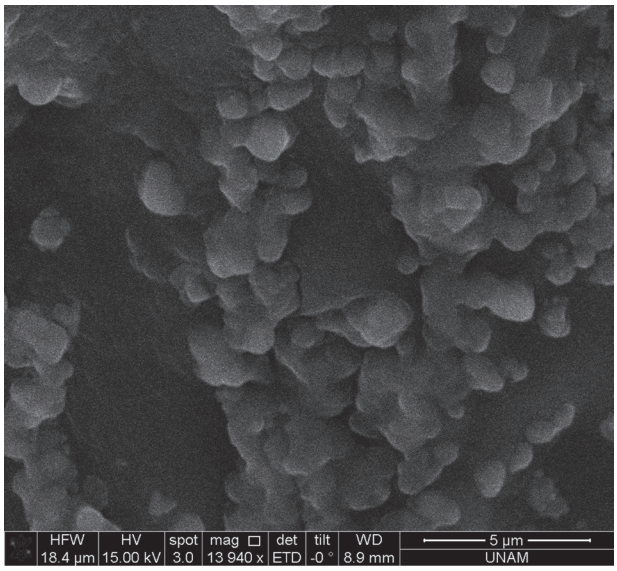



Figure 1. Scanning electron microscope (SEM) views of nanoparticles

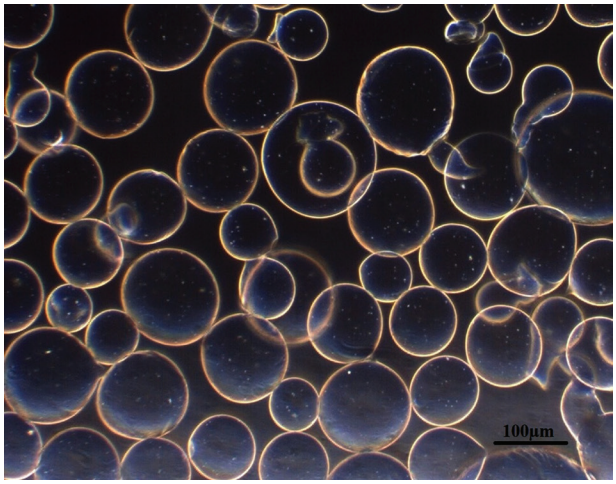



Figure 2. Light microscope views of the microparticles

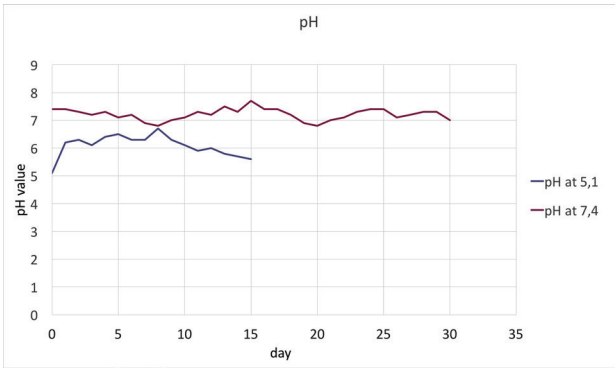



Figure 3. pH values measured from PBS solution during degradation of microparticles

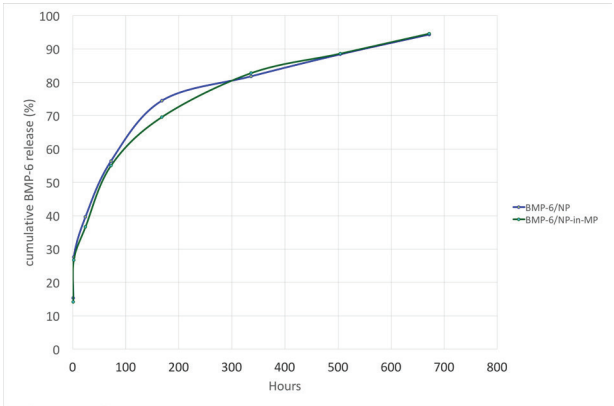
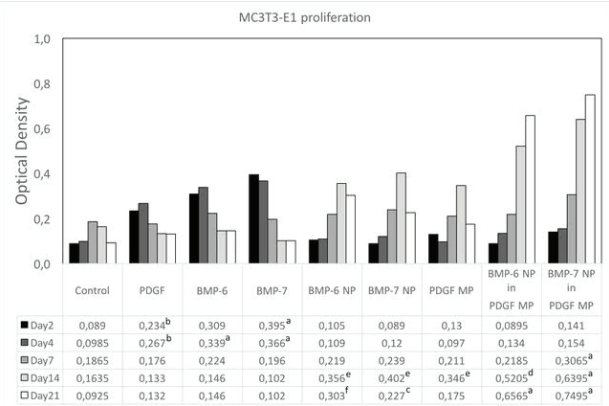



Figure 4. In vitro release behaviour of BMP-6 in two release systems (NPs and NP-in-MP) NP: nanoparticle; MP: microparticle; BMP: bone morphogenetic protein

wide variation (Figure 3). While the physical structure of the particles in the PBS with neutral pH (=7.4) were also similar at different evaluation times, degradation was observed in the particles residing in PBS solution with pH=5.1 at 15 weeks follow-up. Compared to neutral pH, a slight tendency of swelling of MPs was observed in acidic pH.

### *In vitro release studies*

BMP-6 was used in two release systems (NPs and NP-in-MP) to test in vitro release behavior and associated graphics was shown in Figure 4. According to the results, some of the BMP-6 molecules seemed to be localized or adsorbed on the surface of the NPs and were loosely encapsulated relatively. BMP-6 concentrations released from the NP and NP-in-MP systems after 2 h were calculated as 28 and 25 ng/mL, respectively. The highest release period after the burst release (0-2h) was detected between 24 and 72h, where more than 50% of the encapsulated drug passed into solution. The barrier effect of alginate microspheres is most clearly seen in the findings from samples obtained at the end of the first week, indicating that the 75 ng of the encapsulated factor release has occurred whilst MP system only permits less than 70 ng in total. The sustained release of the BMP-6 in that were prepared by incorporating into the NP-in-MP system exhibited a retarding effect through the MP structure surrounding the NPs, especially in 24-72 h time period. After the 24 h release, the concentration of released BMP-6 was calculated as 39 ng/mL and 36 ng/mL for NP and NP-in-MP systems, respectively. The daily released amount between 3rd and 7th days for both systems




| | Control | PDGF | BMP-6 | BMP-7 | BMP-6 NP | BMP-7 NP | PDGF MP | BMP-6 NP in PDGF MP | BMP-7 NP in PDGF MP |
|---|---|---|---|---|---|---|---|---|---|
| Day2 | 0,089 | 0,234[b] | 0,309 | 0,395[a] | 0,105 | 0,089 | 0,13 | 0,0895 | 0,141 |
| Day4 | 0,0985 | 0,267[b] | 0,339[a] | 0,366[a] | 0,109 | 0,12 | 0,097 | 0,134 | 0,154 |
| Day7 | 0,1865 | 0,176 | 0,224 | 0,196 | 0,219 | 0,239 | 0,211 | 0,2185 | 0,3065[a] |
| Day14 | 0,1635 | 0,133 | 0,146 | 0,102 | 0,356[e] | 0,402[e] | 0,346[e] | 0,5205[d] | 0,6395[a] |
| Day21 | 0,0925 | 0,132 | 0,146 | 0,102 | 0,303[f] | 0,227[c] | 0,175 | 0,6565[a] | 0,7495[a] |

**Figure 5.** Inter-group comparison of MC3T3-E1 cell proliferation
a: significantly higher than other groups
b: significantly higher than other groups except BMP-6 and -7
c: significantly higher than PDGF MP
d: significantly higher than other groups except BMP-7 NP in PDGF MP
e: significantly higher than control, PDGF, BMP-6 and -7
f: significantly higher than other groups except BMP-6 NP in PDGF MP and BMP-7 NP in PDGF MP
NP: nanoparticle; MP: microparticle; PDGF: platelet derived growth factor; BMP: bone morphogenetic protein; ($p<0.05$)

were found as 4.5 and 3.5 ng per day for NP and NP-in-MP systems where the approximately 70% of the encapsulated drug was released. At the end of the third week, where released amount is the 90% of the total amount, the daily released amount decreased down to 0.4 ng per day for each system was also found in the activity range.

### *MC3T3-E1 cell culture experiments*

Comparison of cell proliferation of all groups were illustrated in Figure 5.

***Day 2.*** While the control group showed less cell proliferation compared to FS groups (<0.0001), the cell numbers were similar with all particle groups. The number of cells in PDGF group was similar to BMP-6, less than BMP-7 (<0.01) and higher than all particle groups (<0.05). While proliferation in BMP-6 group was similar to BMP-7, it was significantly higher compared to other particle groups (<0.0001). BMP-7 also showed better cell proliferation compared to other particle groups (<0.0001). When particle groups were evaluated with each other, no significant difference was detected at any match.

***Day 4.*** Similar to day 2, control group demonstrated less proliferation than FS groups (<0.001) and it was statistically similar with particle groups. The number of cells in PDGF group was higher than all particle groups (<0.01) except BMP-7 NP in PDGF MP whereas it was also similar to BMP-6 and -7 FS groups. BMP-6 and BMP-7 groups had higher cell numbers than all particle applied groups (<0.0001) but they were statistically similar with each other. Particle groups again did not show any differences when compared among each other.

***Day 7.*** At this day, BMP-7 NP in PDGF MP group reached to highest cell proliferation amount and when compared with all FS groups, the difference was statistically significant (<0.05). BMP-7 NP in PDGF MP also showed higher values than PDGF MP (<0.05). Except those, no significant differences were observed.

***Day 14.*** At day 14, particle groups reached to statistically significantly higher number cells compared to FS groups (<0.0001). While FS applied wells did not show intergroup differences, particle groups containing either BMP-6 or -7 in PDGF MPs demonstrated higher values than the particles loaded with only one type of growth factor (<0.01). When the particles loaded into NP-in-MP system were compared with each other, BMP-7 loaded group showed higher cell proliferation than BMP-6 loaded ones (<0.001).

***Day 21.*** Except the significant difference between BMP-6 NP and others, all particle groups exhibited similar cell proliferation with FS groups (<0.001). BMP-7 NP and PDGF MP applied wells had similar values with PDGF, BMP-6 and

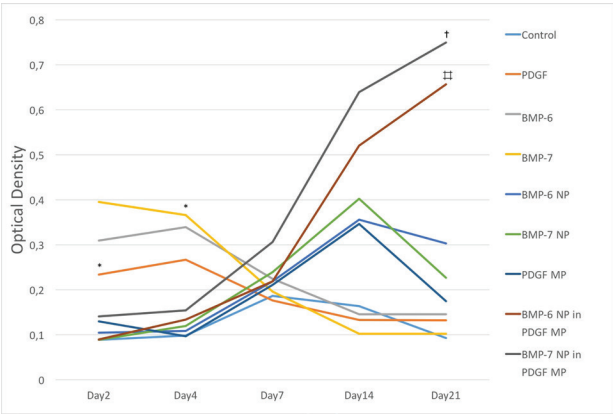



**Figure 6.** Time-dependent change of MC3T3-E1 cell proliferation profiles
*: significantly higher than day 21
†: significantly higher than day 2 and 4
⌗: significantly higher than day 2
NP: nanoparticle; MP: microparticle; PDGF: platelet derived growth factor; BMP: bone morphogenetic protein; ($p<0.05$)

-7 FS groups whereas BMP-6 NP and NP-in-MP groups provided more cell proliferation (<0.0001). Among particle groups, except BMP-6 NP vs. BMP-7 NP and BMP-7 NP vs. PDGF MP, all comparisons revealed remarkable difference (<0.05). The proliferation rates of the wells were ranked from high to low as follows; BMP-7 NP in PDGF MP, BMP-6 NP in PDGF MP, BMP-6 NP, BMP-7 NP and PDGF MP.

When the time-dependent change was considered within groups; PDGF, BMP-6 and -7 groups showed significant reduction after 2nd or 4th day of cultivation (<0.05). On the other hand, both NP-in-MP groups revealed an increase that reach to significance at day 21 in the same time direction (<0.05) (Figure 6).

## DISCUSSION

Regarding to their plasticity, biodegradability, and high biocompatibility, PLGA particles have been widely used in biomedicine as scaffolds or sustained release systems.[26-29] Considerable effort including modification of their dimensions and surface characterization by several engineering strategies has been given to optimize their efficiencies.[30-35] However, these particles have a weakness due to their relatively low loading efficiency and difficulties in controlling the release rate and stability of the proteins/drugs.[26,36-40] In the present study, PLGA NPs were used for BMP loading and according to their characterization results; optimal diameters, zeta potential values and surface morphologies, were detected after their synthesis and loading. The characterization results showed compatibility with the relevant studies[20] and exhibited a favorable NPs for optimal cell uptake and bioactive mediator loading.

Alginate has been widely used for preparing a broad spectrum of drug/protein delivery systems via the use of different manufacturing techniques.[41] The present MP characterization results were compatible with the existing literature showing the effective release systems constructed with alginate MPs.[42,43]

Extracellular pH is a very well-known phenomenon in regulation of osteoblastic cell activity and associated functions of the bone.[44] With the enzymatic activity, degradation products may change the environmental pH that may disturb the balance of cellular activity. In the present study, a major change was not detected by gradual degradation of MPs during 30-weeks follow-up. Accordingly, MPs can be considered as they have a neutral behavior in cell culture experiments.

In the beginning of the follow-up, both NP and NP-in-MP systems showed similar burst release amount. Then, the amount of released BMP-6 decreased in MP surrounded group whereas it protected its effective release amounts until the end of 672 hours period. After the 14th day, the release profiles of both group became similar, indicating the MPs was no longer capable of acting as a barrier coating for NPs, either by degrading or permitting the NP leak by swelling. This result was compatible with the literature comparing FS applications with particle systems[45] or using nano-in-micro systems.[46]

According to the cell culture experiments, GF application by FS method resulted with high MC3T3-E1 cell proliferation in the first days of culture experiments. Afterwards, GFs that applied within the particles provided increasing cell numbers whereas a tendency of reduction was observed in FS applied cells. With the continuity of the follow-up period, the higher proliferation rates were protected in particle-applied wells particularly using the NP-in-MP system.

Both BMP-6 and -7 play significant roles in bone morphogenesis. Moreover, they regulate mineralization events and associated gene expressions.[47] In the present study, besides evaluating the release kinetics of a NP-in-MP system, it was aimed to test the system in an in vitro cell culture experiment by using osteoblastic cells. In addition to cell proliferation, the mineralization and gene expression profiles of the cells could not be tested inside the same experiment model and thus, should be considered as a limitation of the study. The release studies were conducted with only BMP-6 and the absence of release studies including PDGF and BMP-7 in separate or combined systems can be counted as another limitation of the study.

## CONCLUSION

According to the results of the present in vitro study, treatment of osteoblastic MC3T3-E1 cells with the FSs of PDGF, BMP-6 or -7 showed a burst release causing rapid cell proliferation at early stages. when the GFs were loaded inside MPs, release and cell proliferation period was present for relatively longer period. However, when compared to those applications, NP-in-MP system exhibited a gradual and longer time factor release that resulted with delayed but elongated high cell proliferation period. When the cell

cycle in bone formation process was considered, PDGF mainly affect cell proliferation whereas BMPs mainly play the role for cell differentiation. From this point of view, the present system can be considered as a promising system in future for mimicking the natural bone formation process by providing sequential release of PDGF and BMPs, for bone tissue engineering. However, the present experimental design is not enough to verify this effect without evaluation of the mineralization and gene expression profiles of osteoblastic cells and therefore, the present results should be supported with adding such evaluations to different experimental designs testing NP-in-MP release system by including other bioactive mediators.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to report.

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