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Guided bone regeneration by the development of alendronate sodium loaded in-situ gel and membrane formulations

Umut Can Öz[a], Mete Toptaş[b], Berrin Küçüktürkmen[a], Burcu Devrim[a,*], Ongun Mehmet Saka[a], Mehmet Salih Deveci[c], Hasan Bilgili[d], Elif Ünsal[e], Asuman Bozkır[a]

[a] Ankara University Faculty of Pharmacy Department of Pharmaceutical Technology, 06560 Yenimahalle-Ankara, Ankara, Turkey
[b] Bezmialem University Faculty of Dentistry Department of Periodontology, İstanbul, Turkey
[c] Health Sciences University Gulhane Medical Faculty Pathology Department, Ankara, Turkey
[d] Ankara University Faculty of Veterinary Medicine Department of Surgery, Ankara, Turkey
[e] Ankara University Faculty of Dentistry Department of Periodontology, Ankara, Turkey

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ABSTRACT

Biocompatible materials applied in guided bone regeneration are needed to prevent leakage caused by the invasion of peripheral epithelium. The aim of this study is to develop a thermosensitive in situ gel system containing alendronate sodium loaded PLGA nanoparticles and alendronate sodium loaded membranes for guided bone regeneration. Thermosensitive Pluronic F127 gel system was preferred to prevent soft tissue migration to the defect site and prolong the residence time of the nanoparticles in this region. In situ gel system was combined with membrane formulation to enhance bone regenaration activity. Efficacy of combination system was investigated by implanting in 0.5 × 0.5 cm critical size defect in tibia of New Zealand female rabbits. According to the histopathological results, fibroblast formations were found at defect area after 6 weeks of post implantation. In contrast, treatment with the combination of in-situ gel containing nanoparticles with membrane provided woven bone formation with mature bone after 4 weeks of post implantation. As a results, the combination of in-situ gel formulation containing alendronate sodium-loaded nanoparticles with membrane formulation could be effectively applied for guided bone regeneration.

1. Introduction

Guided bone regeneration (GBR) is a widely used procedure in the field of maxillofacial and oral surgery, implantology, and periodontics. This procedure is necessary for isolating surrounding soft tissues from bone tissue, providing a cavity with a source of osteoblasts and a blood supply using barrier membranes (Christgau et al., 1997; Florjanski et al., 2019). Regeneration of critical size maxillofacial and calvarial defects and new bone formation via a synchronised progression of events recapitulating intramembranous ossification can be achieved with GBR. Furthermore, preclinical and clinical evidence suggests that GBR presents a successful therapeutic approach for the treatment of peri-implant bone defects and for the preservation of the dimensions and the configuration of the alveolar socket following tooth extraction ((Retzepi and Donos, 2010). In this procedure, biomaterials are applied to serve as a transient matrix for cell proliferation, and differentiation and mineralization of the extracellular matrix. Biomaterials used for GBR should be biocompatible, provide peripheral sealing by preventing peripheral epithelium and connective tissue invasion, remain stable during treatment, easy to apply, not have surface properties that may cause bacterial retention, and be suitable for sterilization (Buser et al., 1993; Watzinger et al., 2000). Additionally, biomaterials such as membranes used for GBR are needed to keep the structural integrity during 4-6 weeks to prevent soft tissue migration through bone formation area (Caffesse et al., 1994; Galgut et al., 1991; Selvig et al., 1990). From this point of view, PLA/PLGA are very appropriate materials for the application in GBR since the degradation time of membranes made of these polymers keep their physical integrity for 6-8 weeks (Hua et al., 2014; Kim et al., 2009).

Nanotechnology has made it possible to create structures within the same size as those that constitute naturally occurring bone. Hence, nanoparticles can be used to modify scaffolds properties, leading to enhanced characteristics such as superior mechanical properties and osseointegration, osteoconduction, and osteoinduction. Moreover, nanoparticles can be applied to deliver drugs in a controlled manner, either systemically or locally (Couvreur, 2013; Vieira et al., 2017;

* Corresponding author.
E-mail address: bdevrim@pharmacy.ankara.edu.tr (B. Devrim).

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Yohan and Chithrani, 2014).

Polymeric nanoparticles can enable controlled release of the active substance to the target site for days or even weeks. Poly(lactide-co-glycolide) (PLGA) is a biopolymer approved by the FDA as well as the most widely used synthetic polymers in the fields of tissue engineering and drug delivery because of its properties such as being inert in the physiological environment, biodegradable, biocompatible and capable of degradation into non-toxic products. Poly(lactide-*co*-glycolide) (PLGA) copolymers are suitable carriers for the preparation of nanoparticulate systems with many low or high molecular weight, hydrophilic and hydrophobic active agents in different therapeutic activities (Danhier et al., 2012; Martins et al., 2018).

In situ gel forming systems are defined as viscous liquids that are applied as a solution, colloidal sol or suspension, and become gelled when subjected to physiological conditions (pH, temperature, effects of ions) (Nanjawade et al., 2007). In the bone repair procedure, one of the main points of success after placement of the bone substratum that mimics the natural bone structure into the defect is its contact with the natural bone. If the defect is irregularly shaped, it is difficult to contact the bone when the scaffold is used, which is often seen clinically (Lippens et al., 2009). The in-situ gel system is suitable for this purpose because it will take the shape of the defect. In situ gel systems have a solution-like ease of application. Compared to gel formulations, the advantage is that full-dose and reproducible application is possible. Once in situ gel systems are applied to the body, there is no need for removal and patient compliance is good.

Alendronate sodium (AS) is a kind of nitrogen-containing bisphosphonate approved by U.S. Food and Drug Administration, and acts as a potent inhibitor of bone resorption (Carvalho Dutra et al., 2019; Wang et al., 2019). It is confirmed that AS can inhibit osteoclastic bone resorption via GTP associated pathways (Wang et al., 2019). Alendronate sodium (AS) is a clinically well-prescribed anti-fracture drug for the treatment of a wide range of bone disorders (Dong et al., 2018). However, there are many challenges of the application of AS regarding low systemic bioavailability and potential side effects. As a consequence, a local administration of AS is practically required.

This study aimed to develop a thermosensitive in situ gel system containing AS loaded PLGA nanoparticles and AS loaded membranes to use in GBR for the first time. For this purpose, AS-loaded PLGA nanoparticles were prepared by nanoprecipitation method and dispersed in Pluronic F127 based termosensitive in situ gel system. Furthermore, AS-loaded membranes were fabricated for the utilization as a biodegradable barrier formulation that prevents the soft tissue migration through defect area. The in situ gel system containing AS-loaded PLGA nanoparticles and AS-loaded membranes were characterized and their bone regeneration efficiencies were tested on rabbit tibia model during 6 weeks. In the in vivo study, in situ gel formulation containing AS-loaded nanoparticles and AS-loaded membrane formulation were applied either as individually and as a combination.

2. Materials and methods

2.1. Materials

Alendronate sodium (AS) was provided from Mustafa Nevzat Pharmaceutical Company (Turkey). Poly(lactide-*co*-glycolide) (PLGA) (50:50) and chitosan were supplied from Aldrich (St. Louis, MO). Pluronic F127 and Pluronic F68 were purchased from Sigma (St. Louis, MO).

2.2. Methods

2.2.1. Preparation of nanoparticles

In our previous study (Oz et al., 2019), AS-loaded PLGA nanoparticles were prepared by nanoprecipitation method and optimized with central composite design. In this method, nanoparticles are readily obtainable and feasible for scaling up. According to the the optimized formulation; PLGA (187.5 mg) was dissolved in acetone (18.75 ml) as an organic solvent and AS (25 mg) with Pluronic® F68 (125 mg) was dissolved in pH 7.4 phosphate buffer (50 ml). After that, the polymeric solution was added to the aqueous solution and the mixture was immediately stirred until all the acetone was evaporated. After the nanoparticles were formed, concentrated with Vivaspin (Sartorius, 300.000 Da MWCO) and lyophilized.

Table 1
Gelation temperatures of in situ gel formulations.

Pluronic F127 (%)	Chitosan (%)	Gelation temperature (°C)
18	-	31.00 ± 1.00
20	-	28.30 ± 0.50
18	0.25	35.00 ± 2.60
18	0.25	29.67 ± 0.58

The data presented as mean ± SD.

2.2.2. Preparation and characterization of in situ gel formulations containing nanoparticle

Thermosensitive gels were prepared according to the cold method described by Schomolka et. al. (Schmolka, 1972). Solutions of varying concentrations of Pluronic F127 and chitosan were prepared and evaluated for gelling temperature in order to determine suitable compositions for in situ gelling (Table 1). Then, AS-loaded PLGA nanoparticles were added into in situ gels to obtain in situ gel formulations containing nanoparticle.

2.2.3. Determination of gelation temperature

The gelation temperature was measured using the method reported by El-Kamel and El-Khatib (El-Kamel and El-Khatib, 2006). Briefly, two grams of the polymer solution was transferred to a transparent vial containing a magnetic stirring bar. The vial was heated gradually 20°C to 40°C with a constant stirring rate at 125 rpm. The temperature at which the rotation of the bar stopped was taken as gelation temperature.

2.2.4. pH measurement

pH measurements of the in situ gel formulations were performed with SenTix 82 pH electrode (Inolab wtw).

2.2.5. Viscosity

Viscosity measurement of the prepared formulations were carried out on a Brookfield RVTDV-IIviscometer using spindle T-E at 25°C and 37°C. Angular velocity increased gradually from 0.5 to 100 rpm. The average of three readings was used to calculate viscosity.

2.2.6. Preparation and characterization of AS-loaded memrane formulations

Alendronate sodium (AS)-loaded membrane formulations were prepared by solvent casting method (Chitrattha and Phaechamud, 2016; Tamburaci and Tihminlioglu, 2017). During the process, various amounts of poly(*D,L*-lactic acid) (PDLA), triethyl citrate (TEC) and Poloxamer 407 were dissolved respectively in 5 ml of acetone and poured onto PTFE mold. Afterwards, solvent was evaporated at 37°C during 24 hours. 1.2 mg of AS was added to acetone solution to obtain AS-loaded membrane formulations. Composition of AS-loaded membrane formulations were given in Table 2.

The mechanical properties of membrane formulations were analyzed by measuring tensile strength MPa) and elongation at break (%) via Texture Analyzer (TA.XT Plus Texture Analyzer, Stable Micro Systems, UK) equipped with Tensile Grip with a 5 kg load cell and an extension rate of 5.0 mm/min. The membranes were cut into 2 × 2 cm square shaped specimens and tested. The mechanical properties of the

Table 2
Composition of AS-loaded membrane formulations.

Membrane Formulation	AS (mg)	PDLA (mg)	Poloxamer 407 (mg)	TEC (mg)
M1	1.2	400	9.6	5
M2	1.2	400	9.6	20
M3	1.2	400	9.6	40

AS: Alendronate sodium, PDLA: Poly(*D,L*-lactic acid), TEC: Triethyl citrate.

membranes were calculated from the strain-stress curves. Three membrane formulations were tested at least.

M3 coded membrane formulation was used for the animal experiments because of enhanced mechanical characteristics compared to M1 and M2.

2.2.7. Stability

The stability studies were performed according to ICH Q1A guidelines for drug substances intended for storage in a refrigerator at accelerated storage conditions. Formulations were evaluated for stability at 25°C and 60% relative humidity for six months.

2.2.8. Animal experiments

The effect of in situ gel formulations containing AS-loaded nanoparticles and AS-loaded membrane formulations on bone healing were tested on 90 adult female New Zealand white rabbits (1 year old, weight varies 2250-2350 g). The animals were fed with a standard laboratory diet and water and had a 12 h day/night cycle. The rabbits were housed separately in standard-cages in a temperature controlled room. Furthermore, the animal study protocols were approved by the Ethics Committee for Animal Care of Ankara University. Surgery was made under aseptic conditions.

2.2.9. Surgical procedure

General anesthesia was obtained by the administration of ketamine (20 mg/kg) and xylazine (0.2 mg/kg). The skin was cleaned with iodine surgical soaps. An incision of 3-4 cm length was made on the tibia (proximal 1/3 metaphyser-diaphyser transition zone). The bone surface exposed by blunt dissection then 0.5 × 0.5 cm defects were created using a rotary drill. The defects were filled with either the in situ gel formulation containing AS-loaded nanoparticles, the AS-loaded membrane formulation, or the combination of in situ gel formulation containing AS-loaded nanoparticles + AS-loaded membrane formulation. The gel formulations were directly injected in to the defects, and the membrane formulations were positioned on the defects (Fig. 1). The soft tissues are repositioned carefully and sutured with superficial silk sutures. The infection prophylaxis was provided with 20mg/kg/day cefazoline sodium preoperatively and 2 days postoperatively.

The bone defects were treated according to the prescribed conditions for each of the four groups (Table 3) and 6 rabbits from each group were sacrificed at 2^{nd}, 4^{t}h, and 6^{th} weeks after the treatment. Additionally, individual application of in situ gel formulation containing blank (AS-free) nanoparticles (n = 6), individual application of blank membrane formulation (n = 6), and combined application of in situ gel formulation containing blank nanoparticles with blank membrane formulations (n = 6) were evaluated only after 6 weeks to exhibit the effect of local administration of AS on bone regeneration.

2.2.10. Histopathological processing

For histopathological evaluation, the soft tissues on the samples obtained were dissected. Afterwards, the extremity containing only tibia, was left in 10% neutral formaldehyde solution for a 48 hours period for fixation. Then, fast decalcification was made in 10% formic acid in a time period of 3-5 days and the tissues were left in 10% neutral formaldehyde solution for 48 h again. The defects, previously marked on tibia were sampled transversally. The the tissue processing was carried out by a automatic processor. Briefly, each tissue sample was treated with 80 % alcohol for 1 h, 95 alcohol for 1 h (3 changes), absolute alcohol for 1 h (3 changes), xylene for 1 h (3 changes), paraffin for 1 h (3 changes) and paraffin under vacuum for 1 h, respectively then finally embedded into parafffin block manually. After the tissue processing, the histological sections were prepared to a final thickness of 4 microns by microtome and were stained by using hematoxylin-eosine staining (Ekicioğlu et al., 2005) to identify new bone formation. The sections were analyzed by light microscopy (Nikon 80i microscope and Nikon camera with NIS elements basic research system was used for microphotography of tissues) with 100 ×, 200 × and 400 × magnifications. The results were evaluated according to the modified healing scores given in Table 4.

The evaluated bone samples were scored histopathologically based on a scale ranging from lamellar bone formation to bone without any reactions, depending on their healing status. In these stages, inflammation, fibrosis formation, woven bone development and finally lamellar bone development, which means integration to the main bone, patterns were investigated. Bone healing patterns were evaluated according to the scoring in Table 4. The average scores (n = 6) obtained from each specimen were statistically evaluated using ANOVA followed by Bonferroni post-hoc test and presented in Table 6.

3. Results

3.1. Characterization of in situ gel formulations containing nanoparticles

3.1.1. Nanoparticle characterization

Alendronate sodium (AS)-loaded PLGA nanoparticles were prepared using nanoprecipitation method optimized by Oz et al. [20]. The average size of optimum nanoparticle formulation was 71.92 ± 0.98 nm with the polydispersity index (PDI) of 0.14 ± 0.02. The zeta potential of nanoparticles was -18 ± 4.90 mV. The AS encapsulation efficiency was 34.68%.

3.1.2. Gelation temperature and pH

In order to obtain the optimum concentration ratio, various concentrations of Pluronic F127 were investigated (Table 1). A gelation temperature suitable for in situ gel formulations would be 30-36°C according to Kim et al. (Kim et al., 2002). When the concentration of Pluronic F127 was 10-16%, no gelling was observed below 37°C. When the ratio of Pluronic F127 was increased to 18%, the gelation temperature was found to be 31.0 ± 1.0°C. A mucoadhesive polymer chitosan was added to this formulation at a concentration of 0.25%. After the addition of chitosan, the gelation temperature was measured as 35.0 ± 2.6°C. When AS nanoparticles were dispersed in this chitosan-containing in situ gel formulation, the gelation temperature decreased to 29.67 ± 0.58°C and the pH of this formulation was found to be 3.98 ± 0.03.

3.1.3. Viscosity

When the viscosity of the in situ gel formulation containing AS-loaded nanoparticles at 25°C and 37°C was compared, a significant increase in viscosity as a result of gelation at 37°C was observed. As can be seen in Fig. 2, the viscosity decreased with increasing angular velocity and the formulations showed pseudoplastic flow.

3.2. Characterization of AS-loaded membrane formulations

When the mechanical properties of membrane formulations were examined, it was observed that by increasing the amount of TEC used as plasticizer, the tensile strentgh and the elogation at break was increased as shown in Fig. 3. Therefore, M3 coded formulation was chosen as the optimum membrane formulation. In comparison to the tensile strength of the calcium alginate membrane (0.017 MPa) previously developed

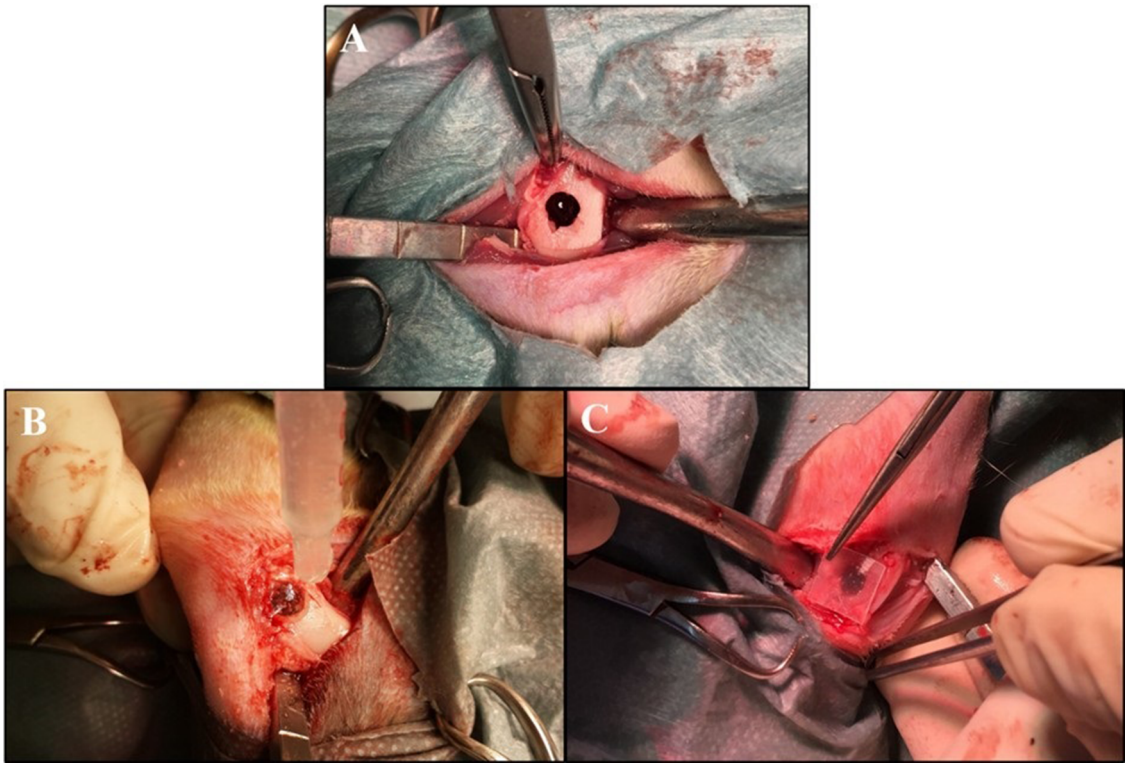


Fig. 1. The image of defect created in tibia (A), application of in situ gel formulation containing AS-loaded nanoparticles (B) and application of AS-loaded membrane formulation (C).

Table 3
Distribution of the test materials in experimental animal groups (n: number of rabbits per group.).

Group	Material	n
1	Untreated control groups	18
2	In situ gel formulation containing AS-loaded nanoparticles	18
3	AS-loaded membrane formulation	18
4	In situ gel formulation containing AS-loaded nanoparticles + AS-loaded membrane formulation	18
5	AS-free blank groups (evaluated only for 6 weeks)	18

AS: Alendronate sodium.

Table 4
Histopathologic evaluation system (modified from Huddleston et al., [40]).

Healing Condition of Defect Area	Scoring
Defect area without any reaction	0
Fibrosis associated with inflammation	1
Fibrous tissue	2
Predominant fibrous tissue with poor woven bone formation	3
Predominant woven bone formation with poor fibrous tissue	4
Focally fibrosis and/or woven bone with lamellar bone formation	5
Only lamellar bone texture (complete healing)	6

for this purpose (Ueyama et al., 2002), our membrane can be considered to have enhanced mechanical properties.

3.3. Stability studies

The stability results of in situ gel formulation containing AS-loaded nanoparticles at 25°C and 60% relative humidity are shown in Table 5. At the end of six months, there was a slight decrease in the pH values of the in-situ gel formulation but no significant change in the gelation temperature. In addition, when the viscosity versus angular velocity graphs are taken into account, there is no significant change in the viscosities of the in situ gel formulation at 25°C and 37°C (Fig. 2).

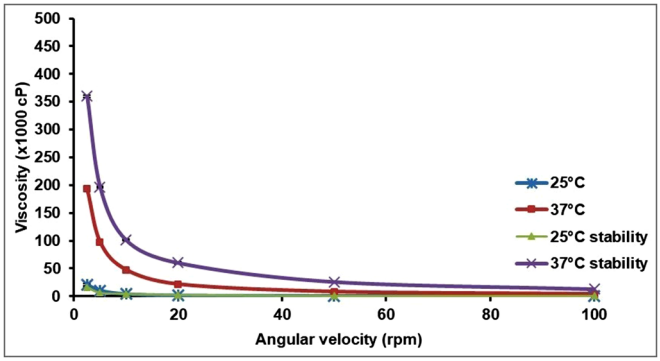
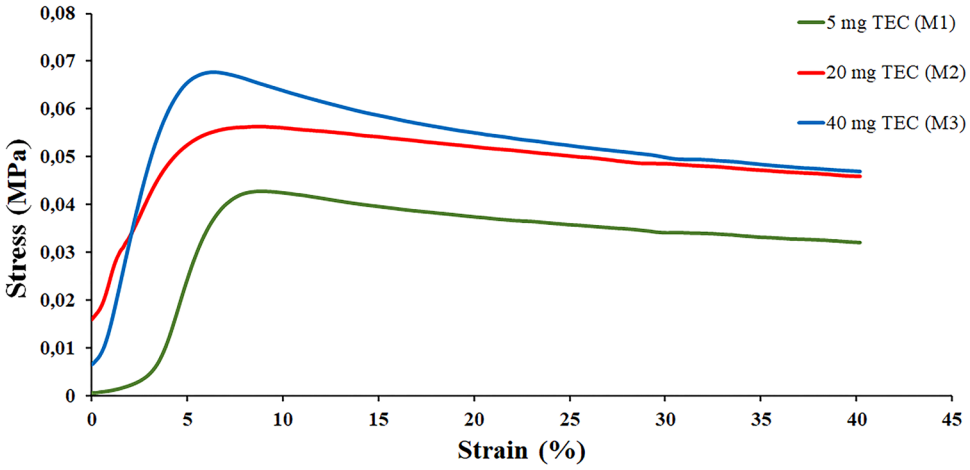


Fig. 2. Viscosity versus angular velocity graphs at 25°C and 37°C and after 6 months stability study.

The M3 coded membrane samples were kept in 25°C and 60% relative humidity conditions for six months. After this 6 months period, tensile strength and elongation at break values were evaluated to assess the changes of mechanical features. While the tensile strength slightly decreased to 0.059 MPa, the elongation at break increased to 9.014%. Although slight alterations were detected, M3 coded membrane was considered to be stable in terms of mechanical properties at the end of 6 months since it was in accordance with previous findings (Tort et al., 2017). Besides, no morphological change was observed during 6 months (Data not shown).

3.4. Histopathological analyses

As shown in Fig. 4A, there was no healing in the untreated control group after two weeks of post surgery. However, fibrous tissue formation was seen as an indicator of the onset of healing when in situ gel



	Tensile Strength (Mpa)	Elongation at break (%)
5 mg TEC (M1)	0.043	6.430
20 mg TEC (M2)	0.065	7.656
40 mg TEC (M3)	0.068	8.878
6 months stability (M3)	0.059	9.014

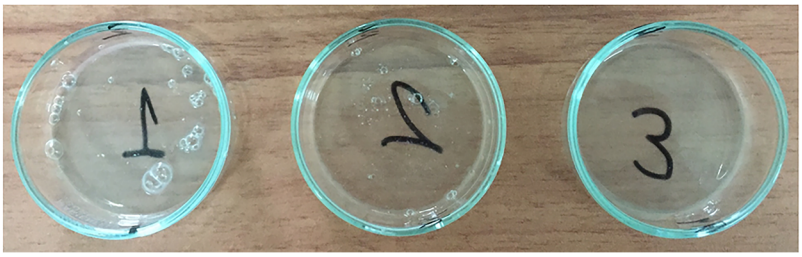


Fig. 3. Mechanical properties of membrane formulations.

containing AS-loaded nanoparticles, AS-loaded membrane and the combination of in situ gel containing AS-loaded nanoparticles and AS-loaded membrane were applied to the defect area. In addition, woven bone formation was observed at the end of the two weeks with the combined application of in situ gel system and membrane formulation (Fig. 4D).

After four weeks of post implantation, combined application of AS containing biomaterials resulted with significantly enhanced bone regeneration than individual application groups and control group ($P < 0.05$). Additionally, AS-loaded mebrane also significantly accelerated the bone genereation than in situ gel system containing AS-loaded nanoparticles ($P < 0.05$) (Table 6). More importantly, lamellar bone formation was observed at the end of only four weeks with combined application which exhibited that the combined application of the developed biomaterials provided the most effective bone regeneration in terms of faster healing compared to individual application of either in situ gel containing AS-loaded nanoparticles or AS-loaded membrane formulation.

At the end of six weeks, no bone formation was observed in the untreated control group. However, woven bone formation was seen in groups which in situ gel containing AS-loaded nanoparticles (Fig. 4B), AS-loaded membrane (Fig. 4C) and combination of in situ gel containing AS-loaded nanoparticles with AS-loaded membrane (Fig. 4D) were applied. Furthermore, AS-loaded membrane formulation and simultaneous application of in situ gel containing AS-loaded nanoparticles with AS-loaded membrane provided lamellar bone formation. However, more extensive fibrous tissue was observed with AS-loaded membrane formulation treatment compared to simultaneous application of in situ gel and membrane formulation.

Besides, we have also checked the bone regeneration for AS free blank groups (individual application of blank nanoparticle containing in situ gel formulation, individual application of blank membrane formulation, and combined application of blank nanoparticle containing in situ gel formulation with blank membrane formulation) only after the 6th week of post implantation to reveal the effect of AS in the formulations. The histological scoring results showed that the AS-loaded formulations were significantly ($P < 0.05$) increased the bone regeneration compared to blank formulations. The data, presented in the supplementary file (Fig. S1), shows that the application of AS loaded nanoparticle containing in situ gel formulation significantly accelerated

Table 5
Six-month stability test results of optimized in situ gel formulation containing AS-loaded nanoparticles.

	Freshly prepared		Six-months later	
	Gelation temperature (°C)	pH	Gelation temperature (°C)	pH
In situ gel formulation containing AS-loaded nanoparticles	29.67 ± 0.58	3.98 ± 0.03	29.33 ± 0.58	3.09 ± 0.02

AS: Alendronate sodium. The data presented as mean ± SD.

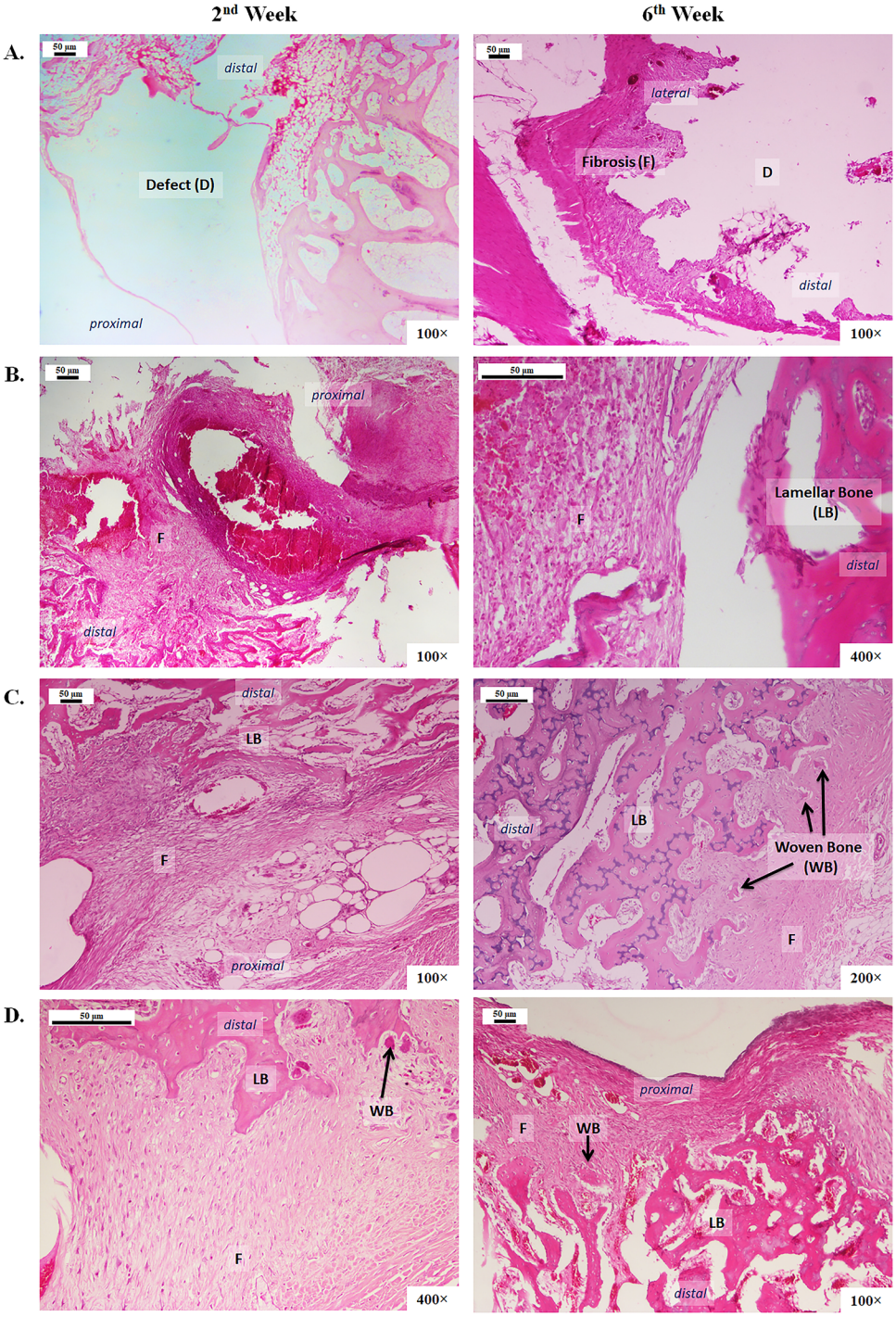


Fig. 4. Histopathological images of defect areas captured with 100 ×, 200 × and 400 × magnifications after 2 and 6 weeks of biomaterial application. A) Untreated cotrol, B) in situ gel containing AS-loaded nanoparticles, C) AS-loaded membrane, D) Combination of in situ gel containing AS-loaded nanoparticles and AS-loaded membrane (D: defect area, F: fibrosis, WB: woven bone, LB: lamellar bone).

the bone repairment process, as the histological scoring is only ~ 2 for the blank group while AS containing application resulted with the score of ~ 4 ($P < 0.05$) (Fig. 5). Similarly, the utilization of AS in the developed materials also significantly increased the bone repairment scoring from ~ 3 to ~ 5 both for the individual membrane application and for the combined in situ gel with membrane applications ($P < 0.05$) (Fig. 5).

However, only the lamellar bone texture, which is the most important indicator of completion of the bone formation (Table 6), was not observed among all the investigated groups.

4. Discussion

Guided bone regeneration (GBR) is an essential procedure in contemporary dentistry. Its main objective is to restore the lost tissues creating an ideal condition to place an implant or maintain a tooth. The GBR involves surgical placement of a cell occlusive biomaterials facing the bone surface, in order to physically seal off the skeletal site in need for regeneration. The development of biomaterials that prevent the migration of undesired cells to the defect site based on bone graft, growth factors, and barrier membranes is necessary for successful bone

Table 6
Histopathological evaluation of the bone healing. ($P < 0.05$ was considered statistically significant, n = 6.).

Week	Untreated control	In situ gel containing AS-loaded nanoparticles	AS-loaded membrane	In situ gel containing AS-loaded nanoparticles + AS-loaded membrane
2	0 ± 0.41 [a]	1 ± 0.41 [a, b]	2 ± 0.41 [a, c]	3 ± 0.63 [a, b, c]
4	0 ± 0.52 [a]	1 ± 0.52 [a, b, d]	3 ± 0.63 [a, d, c]	5 ± 0.52 [a, b, c]
6	1 ± 0.63 [a]	4 ± 0.75 [a, b]	5 ± 0.52 [a]	5 ± 0.41 [a, b]

AS: Alendronate sodium. The data presented as mean ± SD.
[a] $P < 0.05$ for comparison of 'Untreated control' individually with 'In situ gel containing AS-loaded nanoparticles', 'AS-loaded membrane', and 'In situ gel containing AS-loaded nanoparticles + AS-loaded membrane'.
[b] $P < 0.05$ for comparison between 'In situ gel containing AS-loaded nanoparticles' and 'In situ gel containing AS-loaded nanoparticles + AS-loaded membrane'.
[c] $P < 0.05$ for comparison between 'AS-loaded membrane' and 'In situ gel containing AS-loaded nanoparticles + AS-loaded membrane'.
[d] $P < 0.05$ for comparison between 'In situ gel containing AS-loaded nanoparticles' and 'AS-loaded membrane'.

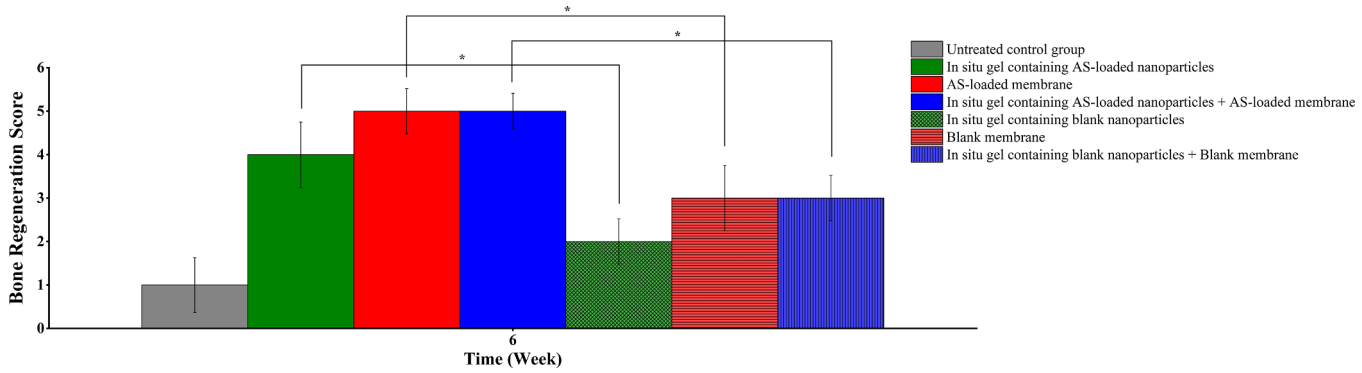


Fig. 5. The regeneration status of the both AS loaded and blank groups, along with control group, at the 6th week after post implantation ($P < 0.05$ was considered as statistically significant, * denotes statistical significance).

regeneration. Biomaterials also create and maintain a secluded space, thus providing an environment to the osteoprogenitor cells, which is permissive for recruitment and proliferation of osteoprogenitor cells, differentiation along the osteoblastic lineage and expression of osteogenic activity (Retzepi and Donos, 2010). In this study, a thermosensitive in situ gel system containing AS-loaded PLGA nanoparticles and AS loaded membranes was developed to use in GBR for the first time. In vitro characterization of AS-loaded PLGA nanoparticles, thermosensitive in situ gel systems containing AS-loaded PLGA nanoparticles and, AS loaded membranes were performed. Then, bone regeneration efficiencies of these biomaterials were tested on rabbit tibia model.

As we previously reported (Oz et al., 2019), nanoparticles smaller than 100 nm were obtained using the nanoprecipitation method. Particle size was significantly ($p < 0.05$) decreased with increasing levels of AS content. Similarly, Posadowska et al. (2015) informed that unloaded nanoparticles were slightly bigger and less uniform than AS loaded nanoparticles. The process of nanoparticle formation became more controlled when AS powder was introduced to the system and this resulted in smaller particles (Posadowska et al., 2015). Furthermore, particle size significantly ($p < 0.05$) decreased with increasing levels of PLGA/Pluronic F68 ratio. It was previously denoted that increasing Poloxamer amount led to an increase in nanoparticle size (Salama et al., 2016). This could be expected due to the accumulation of a larger amount of Poloxamer molecules within the formed nanoparticles and/or on their surfaces. This phenomenon could be attributed to the increase of polymer chains number per unit volume which led to a more bulky structure and thus colloidal system with larger particle size.

Polydispersity index (PDI) is a measure of particle size homogeneity of dispersions and refers to narrow size distribution when smaller than 0.25 (Gindy et al., 2008). In this study, the PDI value of PLGA nanoparticles less than 0.25 indicates that the particle size distribution is narrow. Furthermore, effects of the AS content, PLGA/Pluronic F68 ratio and organic to aqueous phase ratio on PDI were found to be significant ($p < 0.05$).

Zeta potential is an estimation criteria for electrokinetic potential in colloidal systems that particles gain in the dispersed state. When the zeta potential values of nanoparticles are close to ± 30 mV, the colloidal systems are expected to show any aggregation and form stable dispersions that depend on the repulsion forces between particles (Celia et al., 2011; Xie et al., 2011). The zeta potential value of the optimal formulation indicates that the nanoparticles containing AS were not aggregated and formed a stable dispersion. Also, AS-loaded nanoparticles demonstrated a negative zeta potential, which can be attributed to the presence of the ionized carboxyl groups of PLGA on the particles' surface (Le Broc-Ryckewaert et al., 2013).

The encapsulation efficiency of optimal formulation was found to be 34.68%. As stated in our previous study (Oz et al., 2019), AS content and organic to aqueous phase ratio had a positive influence on encapsulation efficiency of nanoparticles. Moradikhah et al. (2020) also reported that % loading efficiency increased with more AS concentration (Moradikhah et al., 2020). Xu and Hanna (2006) denoted that encapsulation efficiencies of BSA to the PLA particles were significantly affected by the organic/aqueous volume ratio. The encapsulation efficiency increased with increases in the organic/aqueous phase volume ratio. This was due to the fact that the viscosity of the emulsion increased with increases in the organic phase ratio. High viscosity inhibited BSA droplet coalescence in the emulsion and the diffusion towards the aqueous phase, both limiting BSA losses.

A gelation temperature suitable for in situ gels would be 30-36°C (Kim et al., 2002). If it was lower than 30°C, gelation would take place. In contrast, if it was higher than 36°C and even the body temperature, the in situ gel would be in a liquid state. The results showed that the gelation temperature range of the in situ gels was 28.30 ± 0.50 to >37°C. When the concentration of Pluronic F127 was less than 18% (10-16%), the sol-to-gel transition temperature was found to be >37°C. However, the sol-to-gel transition temperature decreased with the concentration of Pluronic F127 increased to 18%. The prerequisite for the formation of gels is that the concentration of Pluronic F127 was higher than 16%, because sufficient Pluronic F127 ordered packing was

needed to produce gelation (Jeong et al., 2002). Furthermore, an addition of chitosan caused a slight increase in the sol-gel transition temperature. The sol-gel transition temperature of Pluronic F127 was 31.00 ± 1.00°C, while that of Pluronic F127-chitosan mixture was 35.00 ± 2.60°C. These results could be explained by the fact that the incorporation of chitosan in the formulation can interfere with the formation of micelles in Pluronics and thus amend the dehydration of hydrophobic propylene oxide chain blocks (Sheshala et al., 2019). Furthermore, dispersion of AS-loaded nanoparticles in this in situ gel formulation caused a slight decrease in the gelation temperature. As shown in Table 5, the pH value of the in-situ gel formulation was found to be at physiologically acceptable pH.

Ideally, the in situ gel formulations should have a low viscosity while injecting into the periodontal defect area as the formulation required less force to eject the formulation from the syringe equipped with a needle. After administration, the in situ gel formulation should have high viscosity to contact with bone texture and retain in the defect area for a longer period of time to ensure effective bone regeneration (Ganguly et al., 2017). Therefore, pseudoplastic behavior shown by in situ gel system containing nanoparticles is the desirable property. Similarly, Ruan et al. (2018) have prepared simvastatin thermosensitive gel with pseudoplastic behavior for healing alveolar bone defects (Ruan et al., 2018). The gels' viscosity was markedly decreased when the shear rate was increased, and then it flattened. At high shear rate values, the polymer chains were no longer in a free-stretched state, suggesting a shear thinning behavior of the gels fluid.

The use of a barrier membrane during a GBR procedure is critical to the compartmentalization of the connective tissue growth and new bone growth as well as the prevention of a ridge collapse due to socket void. The lack of a barrier membrane to serve as an occlusive and regenerative aid, can lead to complications with extensive epithelial cell migration into the socket, insufficient bone growth, need for further ridge augmentation, or potential inability for future implant placement (Rodriguez et al., 2018). In recent years, most research groups have focused on the development of membranes for GBR applications that are capable of hindering epithelial tissue infiltration into the periodontal defect and faster bone growth. Membranes were designed to also have the ability to deliver various drugs and biomacromolecules at the defect site, thus further enhancing regeneration. However, bisphosphonates have not yet been used for this application.

Since the non-degradable membranes have disadvantages such as non-resorbability and the need for a second surgical operation, biodegradable membranes including natural and synthetic polymers are commonly used in GBR. Natural polymer membranes have excellent biological properties, such as cell adhesiveness and biodegradability; however, they are characterized by low mechanical strength and short degradation cycle. On the other hand, biodegradable synthetic polymer membranes possess tuned biodegradation, sufficient mechanical strengths, low rigidity, manageability and processability (Wang et al., 2016). Most of current biodegradable synthetic polymer membranes on the market are based on aliphatic polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), poly(hydroxyl valeric acid), and poly(hydroxyl butyric acid), as well as their copolymers (Haidar, 2010). Due to their excellent biocompatibility, controllable biodegradability, low rigidity, manageability, processability, and drug-encapsulating ability, they have been widely considered for GBR procedure.

Clearly, for the clinical success of GBR therapy, it is most important that a balance between rigidity and elasticity be achieved in the synthesis of membranes. Membranes should be rigid enough to bear the compressive forces exerted by the overlying soft tissue and mastication. Meanwhile, a very rigid membrane would not allow for good clinical utilization, due to one has to cut and shape the membrane to adapt to the morphologically distinct periodontal defects (Rowe et al., 2016). In this study, membranes was prepared by using PLA with Poloxamer 407 and TEC. The tensile strength and elongation at break values (Fig. 3) demonstrated that PLA membrane incorporated with Poloxamer 407 and TEC presented comparable or superior mechanical properties to that of a PLA-based membrane. Moreover, increasing the amount of TEC increased the tensile strength and the elongation at break which were in agreement with the results by El-hadi and Alamri in (2018). The enhancement in the mechanical properties of the PLA membranes was attributed to the presence of TEC in the blend. By plasticizing PLA with TEC, it was possible to decrease the stiffness and brittleness and to increase the ductility of PLA as previously reported by Chaos et al. (Chaos et al., 2019).

In order to enhance the GBR approach, simvastatin containing membranes previously tested on animal models and the mature (lamellar) bone formation was observed at the earliest 12 weeks after membrane application (Pişkin et al., 2009).

Drilled holes creating tibial or radial critical-sized femoral defects are traditionally the most commonly used models in rabbits. Many studies have been designed to analyze the confounding factors of bone healing, such as bisphosphonates, taking advantage of bone turnover patterns in rabbits (Chacon et al., 2006; Tsetsenekou et al., 2012). For these reasons, rabbit tibia bone defect model has been used in this study.

There is evidence that certain sex hormones may influence the ability of cells to regenerate tissues (Mehta et al., 2011). Hematopoietic stem cells in mice exhibited differences in cell-cycle regulation according to gender due to higher circulating estrogen. While estradiol increased hematopoietic stem-cell division in both males and females, hematopoietic stem cells of female mice divided more frequently than male cells as a result of higher circulating estradiol (Nakada et al., 2014). Female rabbits were used in this study because of the short healing period in female animals may facilitate the follow-up of the animals.

Size of a critical defect is defined as the smallest size of the intraosseous wound in a particular bone and species of animal which shows less than 10% spontaneous healing during the life time of the animals (Liu et al., 2016). Because the critical size of radial defects was 0.5 cm in some studies that report repair radial defects in New Zealand white rabbits (Ibrahim et al., 2016; Zhao et al., 2020), we created 0.5 cm defects in this study. To reveal the efficacy of in situ gel formulation containing AS-loaded nanoparticles, AS-loaded membrane and combined application of these systems on GBR, the bone formation processes were monitored histopathologically on rabbit tibia bone defect model during six weeks. According to histopathological results, lamellar bone formation which is the most important identifier for the integration to main bone structure, was observed only four weeks after surgery with combined application of AS-loaded membrane and in situ gel system containing AS-loaded nanoparticles. The simultaneous application of these materials is thought to promote bone formation due to reduced osteoclastic activity, presumably by providing full contact of defective bone tissue with AS molecules. However, complete healing could't be observed since six weeks is a short period of time for this evaluation. After six weeks of post implantation, bone regeneration were significantly higher in the all groups compared to control group ($P < 0.05$). The application of in situ gel containing AS-loaded nanoparticles or AS-loaded membrane formulation individually resulted with the formation of predominant fibrous tissue with poor woven bone or woven and lamellar bone formation, respectively. Besides, no bone formation was observed in the untreated control group.

The effect of AS, formulated within developed biomaterials, in the bone regeneration was revealed. Since the bone regeneration investigations of AS-loaded groups was found to be significantly higher than AS free blank groups ($P < 0.05$), we thought that the the utilization of local application of AS would be beneficial in terms of the acceleration the bone regeneration.

No inflammatory reaction was seen in the adjoining soft tissues during experiment. This was an advantage in terms of healing since inflammation would delay healing. The results showed that the most

effective healing was achieved with combined application of in situ gel containing AS-loaded nanoparticles and AS-loaded membrane formulations.

5. Conclusions

In summary, we have developed AS-loaded membrane formulation and in situ gel system containing AS-loaded nanoparticles to GBR for the first time in this study. According to our findings, when in situ gel system containing AS-loaded nanoparticles and AS-loaded membrane formulation were applied simultaneously, lamellar bone formation was observed within such a very short period as 4 weeks. On the other hand, when the formulations were applied individually, predominant fibrous tissue with poor woven bone formation was observed in the AS-loaded nanoparticles containing in situ gel system applied group, and lamellar bone formation could be observed in the group where AS-loaded membrane was applied alone at the end of 6 weeks. In addition, administration of AS via developed membrane and gel based biomaterials with a minimized local dose, is thought to reduced the bone regeneration period in as quick as 6 weeks by inhibiting osteoclastic activity. by reversing the most common side effect (increased bone fracture tendancy) when administered systemically. Overall, these preliminary findings might lead to the development of novel biomaterials and application strategies that could provide enhanced bone formation within a shorter healing period at periodontal applications.

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Credit authorship contribution statement

Umut Can Öz: Funding acquisition, Formal analysis, Investigation, Methodology. **Mete Toptaş:** Funding acquisition, Conceptualization. **Berrin Küçüktürkmen:** Formal analysis, Investigation, Methodology. **Burcu Devrim:** Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Ongun Mehmet Saka:** Formal analysis. **Mehmet Salih Deveci:** Conceptualization. **Hasan Bilgili:** Conceptualization. **Elif Ünsal:** Conceptualization. **Asuman Bozkır:** Funding acquisition, Project administration, Resources, Supervision, Review & editing.

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