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Research Article

Vancomycin-Loaded Gel Ocular Drug Delivery System for Treatment of Endophthalmitis

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ABSTRACT

In case of endophthalmitis, which develops as a result of microbial infection of the intraocular tissues, is not treated, it can lead to anatomical or functional losses in the eye. Intravitreal injections are the most preferred method in the treatment of endophthalmitis, which can be exogenous or endogenous. The combination of antibiotics effective against bacteria has disadvantages such as re-injection, unresponsiveness to treatment, and drug toxicity. Treatment in which antibiotics effective against both gram (+) and gram (-) bacteria are used in combination has disadvantages such as re-injection, unresponsiveness to treatment, and drug toxicity. In order to overcome these disadvantages, studies are carried out to develop injectable forms of active substances that provide long-term release. In this study, the antibiotic Vancomycin (Van), which is frequently used in the treatment of endophthalmitis, was loaded into alginate hydrogels; characterization, in vitro release and toxicity were determined. Its morphology was visualized by environmental scanning electron microscopy (ESEM) and Fourier transform infrared (FTIR) spectroscopy was used to characterize changes in chemical structure. The release of Van from the hydrogels continued for more than 2 weeks. It was determined that the toxicity of free Van decreased with loading of hydrogels. Its antibacterial activity was evaluated with the disc diffusion test and it was determined that it was more effective against Staphylococcus aureus.

1. Introduction

Endophthalmitis is an inflammatory condition caused by bacterial, fungal or parasitic microorganisms infecting the humor vitreous and humor aquosus (intraocular cavity) of the eye. The spread of infection to all tissues of the eye, such as the sclera, tenon capsule and orbital soft tissues, is called panophthalmitis. It can be of exogenous origin after eye surgery, trauma or endogenous origin such as systemic diseases or immune disorders [1]. When endophthalmitis is left untreated, it can result in anatomical and functional loss of the eye [2].

Intravitreal antibiotic injection is the most common treatment method used in the clinic [1]. However, the need for repeat injections in case of prolonged treatment is considered unfavorable both in terms of patient welfare and new complications [3]. Preventive treatment protocols are primarily used for exogenous endophthalmitis. The use of topical antiseptics or antibiotics before a surgical operation, and topical, subconjunctival and systemic administration of a effective antibiotic to ocular tissues during and after surgery reduce the incidence of endophthalmitis. However, in case of bacterial infection, intravitreal, systemic antibiotics and corticosteroids are used in combination with intravitreal drug injection or pars plana vitrectomy [4].

Due to the low and limited permeability of the blood retinal barrier, intravitreal antibiotic administration is frequently preferred in clinical practice for the treatment of endophthalmitis [1]. Antibiotics with broad spectrum and low retinal toxicity are used for treatment. Dual antibiotic

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combinations effective against gram (+) and gram (-) anaerobic bacteria increase treatment efficacy. In clinical practice, Vancomycin and Amikacin/Seftazidime combination is most commonly preferred for this purpose [5]. The use of vancomycin, which is used for Gram (+) microorganisms, at a concentration of 1 mg/0.1 ml has been found to be non-toxic to the retina. There are situations that should be considered in combined use. Since vancomycin has a low pH, it can cause chemical and physical instability when mixed with other antibiotics. Precipitation has been reported after intravitreal injection of vancomycin and ceftazidime combination. This was resolved by complete clearance of the precipitates from the vitreous cavity for 2 months. However, visual acuity decreases during this period [6, 7].

Studies have shown that the administration of toxic, poorly water soluble or short half-life active substances with a carrier increases treatment efficacy. The use of structures such as nanoparticles, nanogels, hydrogels or inclusion complexes eliminates these disadvantages of active ingredients [8, 9]. The vitreous structure of the eye is transparent and heterogeneous, containing 98% water, 2% collagen and hyaluronic acid [10]. Therefore, it is important to choose gel forms that are suitable for the structure of the vitreous, since the non-transparent form of the materials to be applied intravitreal affect the visual field. The use of nanogels or injectable hydrogels as carriers in ocular applications has the advantage of both not affecting the visual function and having different hydrogel structures.

Hydrogels are 3-dimensional structures cross-linked by physical or chemical bonds and swell by absorbing water and biological fluids. Hydrogels are preferred for eye applications due to their high water content and their ability to release by responding to pH, temperature and ionic strength changes in the organism according to the material they are prepared. Hydrogels are soft, flexible and some forms have an injectable structure owing to the high amount of water in their structure. The most important areas of use are controlled drug release systems, wound dressing materials, soft tissue substitution and contact lenses [11-13]. The use of hydrogels for controlled release systems aims to prolong the residence time of the drug in the environment and accordingly extending the application interval, reduce toxic effects and deliver the drug to the target tissue [14].

Hydrogels can be obtained from natural polymers such as cellulose, pectin, starch, dextran, alginate, chitosan, collagen, gelatin or synthetic polymers such as polyesters (lactic acid-glycolic acid copolymers, poly (ecaprolactone), poly (trimethylene carbonate), poly (trimethylene carbonate), poly (orthoesters), etc.), polyamides, poly (glutamic acid), poly (orthoesters) [15, 16]. In this study, hydrogel was prepared from alginate for controlled release of vancomycin. Sodium alginate is a linear polysaccharide (heteropolymer of 1,4-bonded-D mannuronic acid and 1,4-bonded-L-guluronic acid) typically derived from brown seaweed or Azotobacter and Pseudomonas bacteria.

Alginate has different and intensive usage areas in biomedical applications (drug release, implantation and cell encapsulation studies) with its biocompatible, hydrophilic, mucoadhesive, low cost and gelation with divalent cations such as Ca^{+2} (Kuo and Ma, 2001). Homogeneous gel structures are obtained by replacing sodium ions in the structure of sodium alginate with divalent cations such as calcium. While it allows diffusion of the loaded drug due to its large pore size, the pore size can also be adjusted with various modifications [17, 18].

In the study, vancomycin-loaded alginate hydrogels (Van-Alg Gel) were prepared to ensure the controlled and prolonged release of the active ingredient vancomycin in the vitreous. In this way, it is aimed to reduce the frequency of intravitreal injection of vancomycin. After the characterization of Van-Alg Gels, the release profile and toxicity were determined. Since vancomycin is used for the treatment of endophthalmitis, its antibacterial effect was determined by disc diffusion method.

2. Material and Methods

2.1. Materials

Vancomycin loaded into gels and Alginat were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). In vitro cytotoxicity tests were performed with the human retinal microvascular endothelial (HRMEC) (ACBRI 181) cell line. The media and supplementary materials used for the culture of HRMEC cells were obtained from Cell System.

2.2. Preparation of Alg and Van-Alg hydrogel

An alginate-based in situ gelling system has been developed for the controlled release of vancomycin antibiotic [19]. In the in situ gelling system, hydrogel network formation and encapsulation of the active substance into the gel occur simultaneously. 25 mg of Van was added to 5 ml of 1% alginate solution, and the solution was stirred until clear (1 hour). Then, 1% $CaCl_2$ was added dropwise to the solution and centrifuged at 3000 rpm for 5 minutes to remove the Van that was not trapped in the gel after gel formation. The same procedure was followed for the preparation of blank anginat gels, except that PBS was added to the solution instead of vancomycin.

2.3. Characterization of the hydrogels

2.3.1. Morphology

The morphology of the Alg and Van-Alg Gels was determined by using environmental scanning electron microscopy (ESEM) (FEI QUANTA 200 FEG ESEM). Unlike the scanning electron microscope, ESEM is used for imaging uncoated or wet samples. After the gels were placed in the sample holder, surface imaging was performed under low vacuum.

2.3.2. FTIR analysis

Fourier transform infrared (FTIR) (Thermo Fisher, Nicolet is50) spectroscopy was used to characterize changes in chemical structure as a result of vancomycin loading into hydrogels. The spectra of alginate, vancomycin, Alg and Van-Alg Gels were scanned between 500 and 4000 cm-1.

2.3.3. Rheological properties

The rheological properties of the hydrogels were determined using a Malvern Kinexus Pro+ rheometer (Malvern, UK) at 4 and 37°C. In order to evaluate the flow properties and viscoelastic behavior of the hydrogels, shear rate g=0.1–1000 s-1 and frequency scanning tests were determined as 0.1500 Hz. The samples were placed on the plate and measurements were made at a distance of 1 mm between the two plates.

2.4. Vancomycin loading and release

In order to determine the amount of Van loaded on the gels and released depending on time, first of all, spectrum scanning of Van was performed and the wavelength at which it gave maximum absorbance was determined as 280 mn. The amount of vancomycin loaded on the gels (Entrapment Efficiency-EE) was determined by the indirect method. The indirect method is based on the determination of vancomycin remaining in the supernatant after gel formation is complete. The percentage encapsulation efficiency was calculated by subtracting the vancomycin that was not entrapped in the gel from the vancomycin initially added. For release studies, 6 mg of Van-Alg Gel (average of 50±3,5%) was placed on the dialysis membrane (cut-off 14-12 kDa; Sigma, USA) and both ends of the membrane were sealed. The delivery system placed in 10 ml of BSS (Balanced Salt Solution) was incubated at 37±1°C and 100 rpm shaking speed. At certain time intervals, 1 ml of BSS was taken and replaced with the same volume of BSS. Time-dependent release of Van from the gels was determined by using a UV spectrophotometer at 280 nm and all experiments were repeated three times.

2.5. Cytotoxicity

In vitro cytotoxic effects of Van, Alg and Alg-Van Gels were determined by MTT assay on HRMEC cells. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoluim bromide; Thiazolyl blue) or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

assays are performed to determine the cytotoxic effect of materials designed to interact directly or indirectly with living tissue on cells. Metabolically active cells reduce the tetrazolium salt to purple colored formazan crystals. The density of formazan crystals is used as an indicator of cell viability, proliferation, and cytotoxicity [20]. For the MTT assay, HRMEC cells were seeded in a 96-well plate at $5x10^4$ cells/well in 200 µl of medium and incubated overnight in an incubator. Hydrogels prepared at different weights were sterilized by exposing to UV light for 1 hour [21]. Hydrogels (25, 50, 100, 200 µg/ml) were incubated with cells at 5% CO_2 and 37°C for 24 hours. After incubation, the hydrogels were removed and MTT solution (1 mg/ml) was added to the cells. After incubation for 4 hours in the dark and at 37°C, the MTT solution was removed and the plate was read at 570 nm after incubating with pure isopropanol containing 0.04 M HCl for 30 minutes. The cells in the first column were incubated with the medium alone and the average of absorbance values obtained from these wells was accepted as 100% and the cytotoxicity rate was determined by comparing the average of absorbances obtained from the test wells.

2.6. Antibacterial efficacy

The antibacterial effect of Van, Alg and Alg-Van Gels was determined by disk diffusion method. *Escherichia coli (E. coli)* (ATCC 35218) (gram-negative) and *Staphylococcus aureus (S. aureus)* (ATCC 29213) (gram-positive) strains were used for the antibacterial activity of the gels [22]. First of all, bacterial solutions were prepared by suspension method. For bacterial suspensions, colonies of *E. coli* and *S. aureus* were taken from agar plates with inoculating loops and suspended in Todd Hewitt broth. After 24 hours of incubation at 37°C and a shaking speed of 100 rpm, the bacterial solution was diluted with sterile buffer (0.5 McFarland). After inoculating bacteria on agar, discs with a diameter of 6 mm were placed and gels of different formulations and free Van were absorbed into the discs. The dry weights of the Alg and Van-Alg hydrogels were determined. The hydrogels were placed on disks with the weight written on the petri dishes (25, 50, 100 and 200 µg/ml). After 24 hours of incubation of the agar plates at 37°C, the diameters of the zones formed around the discs were measured (n=3).

3. Results and Discussion

3.1. Characterization of hydrogels

3.1.1. Morphology of hydrogels

The change in the morphology of Alg hydrogels caused by vancomycin encapsulation was visualized by ESEM. Hydrogel images taken without drying and coating are given in Figure 1. It is seen that Alg hydrogels have a rough and homogeneous surface, while the pore structure is very low (Figure 1A). However, interconnected micro and macropores are observed in Van-Alg Gel (Figure 1B).

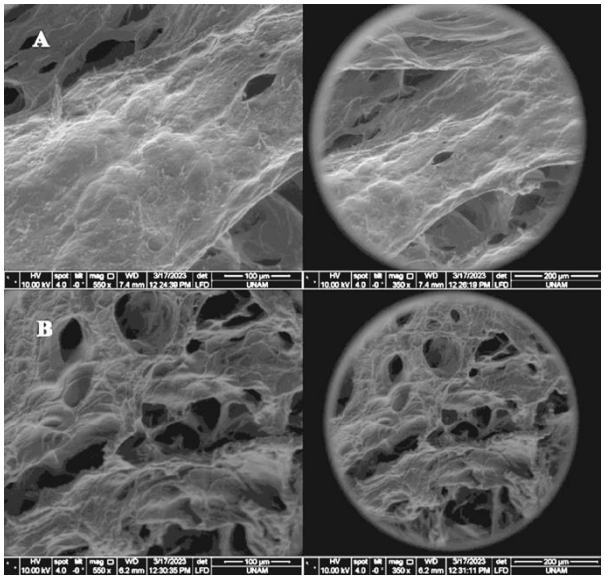

Figure 1. ESEM image of Alg hydrogels (A) and Van-Alg hydrogels (B)

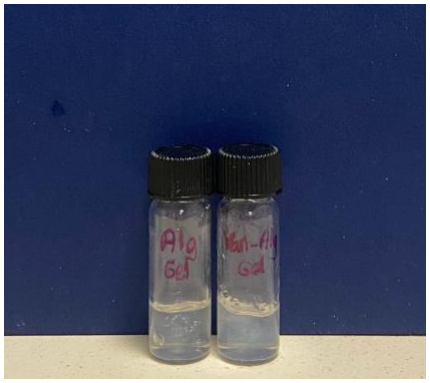

Figure 2. Alg and Van-Alg hydrogels

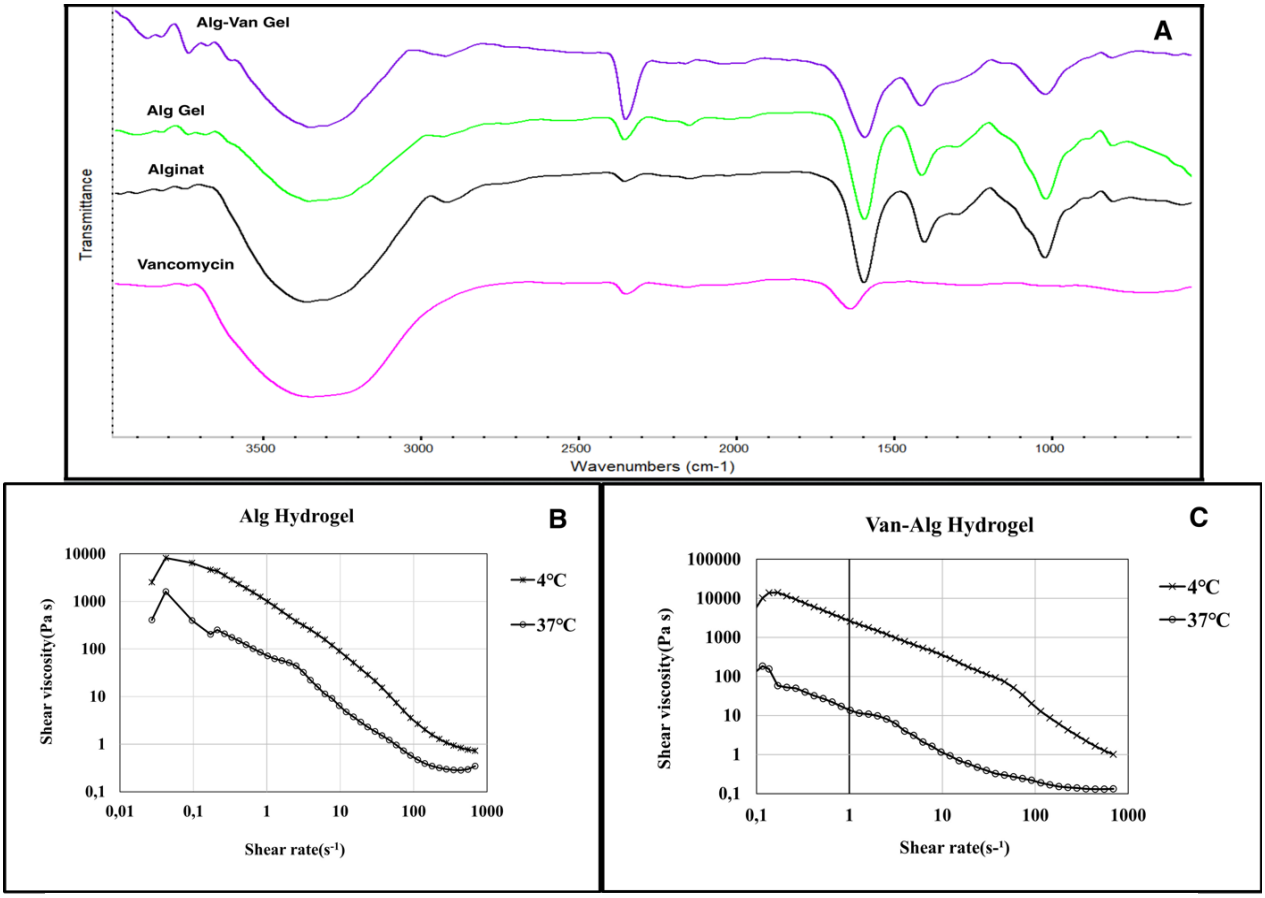

Figure 3. FTIR spectra of Alg and Alg-Van Gels, Alginate Polymer and Vancomicin (A). Viscosity measurement of Alg Gel (B) and Van-Alg Gel (C) at 4°C and 37°C.

This indicates that the active ingredient loading accelerates the formation of the porous structure in the gel structure. When the drug is loaded into hydrogels, their pore structure becomes larger and heterogeneous. The varying microstructure and swelling behavior of hydrogels depending on the polymer or crosslinker concentration have a significant effect on the controlled drug release and drug kinetics [23]. The gel form of the structures to be used in intravitreal applications has the advantages of being compatible with the vitreous structure and minimally affecting the visual field (Figure 2).

3.1.2. FTIR analysis

The peaks obtained from the FTIR spectrum of Alg, Alg Gel, Alg-Van Gel and Van are shown in Figure 3A. The bands at 1591 cm^{-1} and 1415 cm^{-1} are attributed to asymmetric and symmetric stretching vibrations of the carboxylate groups (COO−) found in Alg and Alg Gel. Saccharide ring structure peaked in 1022 and 1295 cm^{-1} bands. C-O and CO-C vibrations of groups in guluronic and mannuronic units peak at 1022 cm^{-1} and 1295 cm^{-1}. The peak in the 814 cm^{-1} (C-O vibration) band is due to the α-configuration of the guluronic units [24-26]. The peaks seen at 3340, 2353, 1642 and 691 cm^{-1} in Van's FTIR spectrum are due to the OH group of H2O, alkinene, C=O group of amides and amines [27, 28]. These characteristic peaks were also seen in the FTIR spectrum of Alg-Van Gel.

3.1.3. Rheological properties

The viscosity of Alg and Van-Alg Gels was measured using a rheometer equipped with a parallel plate (Malvern Kinexus, Japan). The measurements were conducted at temperatures of 4 and 37°C with a gap of 5mm between the plates and a shear rate ranging from 0.01 to 1000 s^{-1} (Figure 3B-3C). Figure 3 shows that increasing the temperature resulted in a decrease in viscosity for both gels. However, the viscosity of Van-Alg gels decreased more compared to Alg gels 37°C. This suggests that Van penetrated the alginate polymer chains, and no molecular interaction occurred between the Van and the polymer chains. The lack of interaction between Van and

the polymer chains affects the drug release profile and explains the initial rapid release of Van.

3.2. Vancomycin loading and release

In the treatment of endophthalmitis, administration of antibiotics with a carrier to reduce the frequency of repetitive injections may increase treatment efficacy. Therefore, the determination of the time-dependent release of vancomycin loaded into alginate hydrogels is necessary to determine the application dose and re-injection time. The following formulation was used to determine entrapment efficiency and loading efficiency. EE was determined that an average of 30±8 % of the initially added drug was loaded into the gels. The Van loaded to the unit weight of the hydrogels is an average of 50±3,5 % (LE). Van release showed a burst effect at the beginning and an average of 70% of the Van was released in the first 24 hours. After 48 hours, the release slowed and remained at a constant concentration (Figure 4A). Van release continued for an average of 2 weeks (average of 80±2,6%).

Entrapment Efficiency = Total Drug conc. – Supernatant Drug conc.) / (Total Drug conc.) x 100

Loading Efficiency (%LE) = Amount of Van in Gel/Amount of Gel × 100.

There is no standard number of injections for an infected eye in clinical practice. Vancomycin and Ceftazidime combination is used in the treatment of endophthalmitis. Imamura et al. studied the pharmacokinetics of intravitreal vancomycin and ceftazidime in Macaque eyes and determined that the half-life of vancomycin in the aqueous humor of normal, vitrectomized, and silicone oil-filled eyes was 29.4, 21.1, and 6.8 hours, respectively [6]. The half-life of vancomycin is 30 hours in average healthy aqueous humor. This period becomes shorter in treated eyes. The patient's need for re-injection is determined by clinical examination and the injection is repeated if the infection remains untreated. However, repeat injections have disadvantages. In long-term release forms, treating the infection with the effect of the first burst effect and continuing the release for at least 1 week ensures treatment effectiveness and thus aims to reduce the number of injections.

3.3. Cytotoxicity

The cytotoxicity of different concentrations of Alg, Van-Alg Gels and free Van on HRMEC cells was determined by MTT assay (Figure 4 B). HRMEC cell viability decreases due to increased concentration of gel and free Van. It has been determined that the toxicity of the empty and Van-Alg Gels is on average over 80% at 200 µg/ml and lower concentrations. Van has higher toxicity on cells. The viability of cells exposed to 100 µg/ml Van and 200 µg/ml Alg-Van Gels were 74.93±4.7% and 82.39±4.37%, respectively ($P<0.05$).

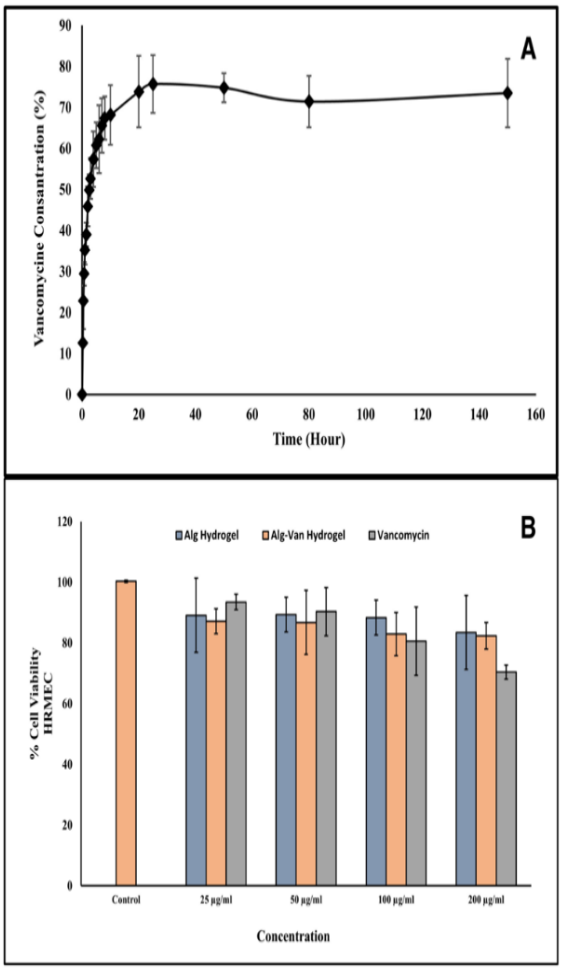

Figure 4. Cytotoxicity of different concentrations of alginate, Van-Alg hydrogel and free Van on HRMEC cells (A). *Van was used at the concentration loaded on gels (50%). Cumulative Van release from Alg Hydrogel (B)

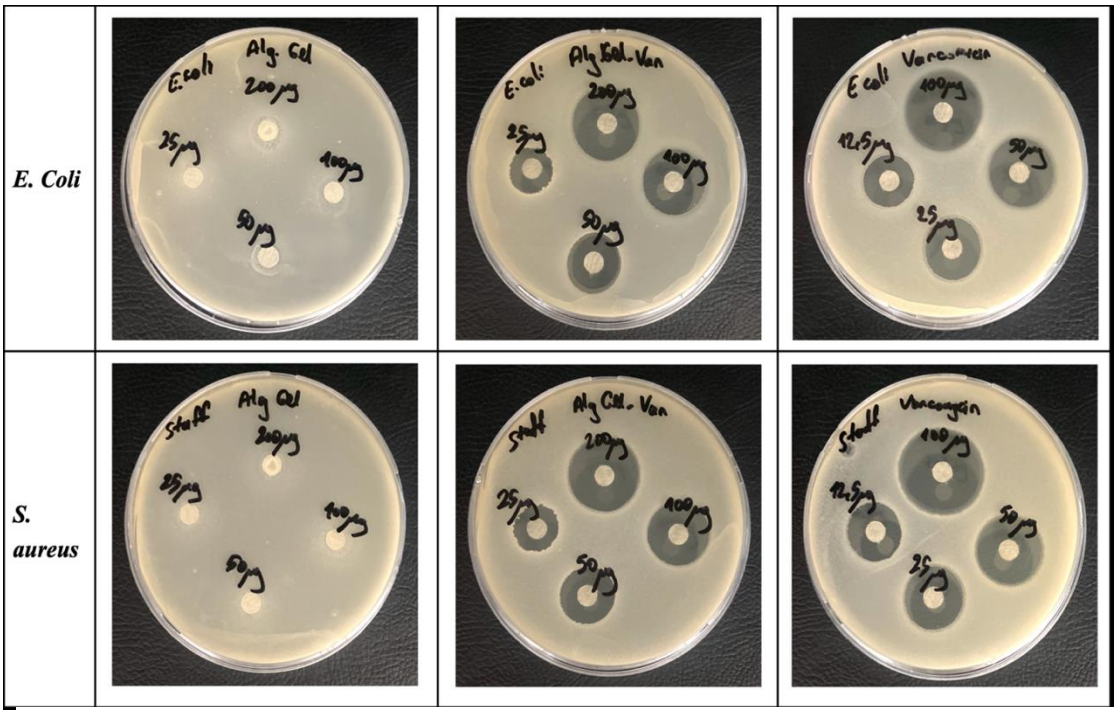

Figure 5. Antibacterial effect of different concentrations of Alg, Van-Alg Gels and Van on E. coli (gram-negative) and S. aureus (gram-positive) strains.

Van causes a decrease in cell viability more than the hydrogel-loaded form. Similarly, in the study conducted in 2019, van's liposome van. In a study conducted in 2019, it was also stated that vancomycin is more toxic than liposome-loaded vancomycin [29]. Cell viability incubated with 25 µg/ml Alg-Van Gels was 87.15±4.14, while it was 82.39±4.37 at 200 µg/ml ($P>0.05$). In the study of Miyake et al in 2019, the toxic and inflammatory effects of moxifloxacin, cefuroxime and vancomycin on human retinal vascular cells (RVEC) were determined. In the study, in which both in vitro and in vivo evaluations were made, retinal toxicity caused by intravitreal injection was evaluated. It has been determined that intravitreal injections of cefuroxime and vancomycin in mice cause retinal and vascular toxicity extending to the inner nuclear layers [30].

3.4. Antibacterial efficiency

The antibacterial effect of Van, empty and Van-Alg Gels was determined by disk diffusion method. Vancomycin is an antibiotic that is widely used in the treatment of endophthalmitis, especially against gram (+) bacteria. Therefore, determination of its antibacterial activity is important in terms of administration and dosage determination. Vancomycin loaded on alginate hydrogels acts by binding to the D-Alanyl-D-alanine terminal in the cell wall of bacteria and inhibiting cell wall synthesis. The glycopeptide antibiotic also disrupts RNA synthesis and bacterial cell wall permeability [31].

Due to this principle, its effectiveness on gram-positive bacteria is higher [32]. It is used in the clinic especially for the treatment of endophthalmitis caused by gram-positive bacteria such as *S. aureus* [33]. Disk diffusion method was used to determine its antibacterial activity. Zone diameters formed by Van and hydrogels at different concentrations against 2 different bacterial species were determined (Table 1 and Figure 5). Alg hydrogels did not form zones in either bacterial species. Van was applied to the gels at the concentration at which it was loaded. Zone diameter formed by Van is higher than Alg-Van Gels ($p>0.05$). In *S. aureus* culture, 200 µg Alg-Van hydrogel and Van has a zone diameter of 2.4±0.2 cm and 2.7±0.3 cm. Vancomycin is more active against gram (+) bacteria. However, this is more evident at higher concentrations.

Table 1. Antibacterial effect of different concentrations of Alg, Van-Alg Gels and Van on *E. coli* (gram-negative) and *S. aureus* (gram-positive) strains.

		E. Coli	*S. aureus*
Vancomycin	**12,5 µg**	1.6±0.3cm	1.6±0.2 cm
	25 µg	1.8±0.2cm	1.9±0.1 cm
	50 µg	2.0±0.3cm	2.2±0.2 cm
	100 µg	2.5±0.2 cm	2.7±0.3 cm
Van-Alg Gels	**25 µg**	1.1±0.2 cm	1.2±0.4 cm
	50 µg	1.4±0.2 cm	1.8±0.1 cm
	100 µg	1.8±0.1 cm	2.1±0.2 cm
	200 µg	1.9±0.2 cm	2.4±0.2 cm

4. Conclusion

Endophthalmitis is an infectious disease that develops when the intraocular cavity becomes infected as a result of surgery, trauma or a systemic disorder and can cause blindness. Although the incidence decreases with preventive measures, it can lead to serious and irreversible indications in the eye. The biggest problems of the combined antibiotic treatments applied are that they require frequent injections and retinal toxicity. Infection can occur weeks after the operation. Therefore, controlled release forms that allow antibiotics to remain in the vitreous for a long time are being investigated. Loading the vancomycin into the hydrogel both prolonged the release time and reduced the toxicity. In addition, its gel form is similar to the vitreous structure. Hydrogel-based controlled release systems show promise as carriers for intravitreal drug administration. The controlled release of 2 antibiotics by loading the hydrogels will increase the effectiveness of the treatment.

Article Information Form

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The Declaration of Conflict of Interest/ Common Interest
Financial interests: Author Ebru ERDAL declare that they have no financial interests.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics
During the writing process of our study, the information of which is given above, international scientific, ethical and citation rules have been followed, no falsification has been made on the data collected, and Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered. I undertake that I have full responsibility and that this study has not been evaluated in any academic environment other than Sakarya University Journal of Science.

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