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ANTIBIOTIC-LOADED DENDRIMER HYDROGELS IN PERIODONTAL BONE REGENERATION: A FEASIBILITY STUDY

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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Abstract

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Purpose: Prescription of a complete course of oral antibiotics following bone grafting procedures is a common clinical practice in surgical periodontics. Prophylactic administration of antibiotics reduces the risk of surgical site infection and systemic administration is preferred over local delivery because of short duration of action in the latter circumstance. The goal of this study is to demonstrate the ability of dendrimer hydrogels (DH) to prolong the release kinetics of antibiotics *in-vitro*. A secondary goal will be to analyze the effect of different particulate bone allografts on the release kinetics of the antibiotic.
Methods: Dendrimer hydrogels (DH) were synthesized from polyamidoamine (PAMAM) G5 and polyethylene glycol diacrylate (PEG-DA) to contain Cefazolin, a first-generation cephalosporin antibiotic, for these *in-vitro* experiments. Two different types of allogeneic bone grafts (demineralized freeze-dried or freeze-dried) along with a negative control (no bone graft) were used to study the effects of bone graft on the release kinetics of cefazolin. Samples were bathed in PBS and incubated at 37^{o} Celsius while 1mL aliquots were taken at time points 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 12hrs, 24hrs, 48hrs, 72hrs. Aliquots were analyzed using HPLC and a standard curve was used to determine the concentration of cefazolin in each sample.
Results: The estimated maximum concentration of cefazolin in samples containing DH was 36.97mcg/mL (95% CI: 34.58-39.36) with 50% released in 4.17 hours (95%: 3.26-5.07) and an estimated growth rate of 0.27 (95% CI: 0.17-0.37). For samples without DH, estimated maximum concentration of cefazolin was 167.4mcg/mL (95% CI: 160.4-174.4) with 50% released in 2.36 hours (95% CI: 2.05-2.67) and an estimated growth rate of 0.70 (95% CI: 0.54-0.87). Bone grafts did not significantly affect the release of cefazolin in this experiment.
Conclusion: DH is a promising platform for long-term release of cefazolin *in-vitro*.

Introduction

Before dental implants were accepted in routine dental practice, teeth lost due to caries, periodontal disease or some other dental malady had to be replaced by fixed or removable prostheses supported by the adjacent teeth. These prostheses often required sacrificing of healthy tooth structure and additional masticatory forces on the abutment teeth. In patients that were completely edentulous, complete prostheses were supported by the oral soft tissue alone, deriving their stability from the negative pressure created by a precise fit to the soft tissue. These dentures took time for the patient to learn how to use effectively and often left the patient with unsatisfactory denture retention, especially in the mandible where the denture was easily dislodged by the movements of the lingual musculature.[1] In the last two decades of the twentieth century, osseointegration of titanium dental implants gained widespread acceptance and gave patients another option to replace their missing teeth and to support previously tooth or tissue-borne prostheses.[2] Today, over 3 million dental implants are placed each year in the United States. In 2019, the total US dental implant market was valued at over $900 million dollars, and is forecast to exceed 1.5 billion dollars by 2027. [3] Osseointegration of dental implants is the process by which dental implants placed within bone transition from mechanical stability during placement to biological stability after weeks-months of healing. This biological stability is due to a direct contact between newly formed bone and the titanium (or more recently titanium alloy or zirconia) surface of the dental implant. After many years of research, osseointegration and long term maintenance of dental implants has become a predictable procedure given proper case selection, with a recent retrospective study showing an overall success rate of 88% and survival rate of 97% after a mean of 5.8 years of service. [4]

In reference to proper case selection for dental implants, one important factor that determines their success is that they are completely surrounded by bone such that they remain osseointegrated. This is important not only during early osseointegration where fibroblasts and epithelial cells can inhibit osseointegration, but also during long term maintenance. Most commercially available implants today have rough surfaces to enhance their early osseointegration because osteoblasts adhere to rough surfaces more readily. During long term maintenance, if these rough surfaces become exposed to the oral microbiome, they become a perfect ecological environment for bacterial pathogens to colonize, causing an inflammatory response which can lead to periimplant bone loss and implant failure. Therefore, when placing dental implants, it is important to have at least 1.5-2mm of bone circumferentially around the implant.[5,6] This is especially important on the buccal aspect of the implant where the labial cortical plate has no intrinsic blood supply and derives its nourishment from adjacent periodontal ligaments, periosteum and endosseous bone marrow. During implant placement, reflection of full thickness mucoperiosteal (gingival) flaps severs the periosteal blood supply to the labial cortical plate. In addition, if the dental implant is placed within 2mm of the labial ridge, the implant will cut off the remaining blood supply from the endosseous bone marrow and the remodeled buccal will be exposed to the oral soft tissue, compromising success.[6]

Not all edentulous sites have adequate alveolar ridge dimensions to accommodate a dental implant. Some patients may have inadequate ridge dimensions genetically, while others may develop these ridge deficiencies over time. Common sources of such ridge deficiencies may be periodontal disease, ridge remodeling after an extraction (which removes the blood supply from the periodontal ligament), or pneumatization of the maxillary sinus. If not properly managed, the alveolar ridge can undergo drastic dimensional changes after tooth extraction.[7] In all such cases,

the alveolar ridge that has inadequate dimensions must be augmented to have adequate dimensions to fit a dental implant if the site is planned to be restored with an implant. Augmentation of the alveolar ridge can be accomplished through a variety of techniques such as guided bone regeneration, block grafting, ridge splitting, distraction osteogenesis, or the maxillary sinuses can be augmented to increase the height of alveolar bone in the maxillary posterior through lateral window sinus augmentation or transcrestal approach. No matter the technique, the goal of therapy is to increase the dimensions of the alveolar ridge by placing a bone graft into the defect site and allowing host bone to encapsulate or replace the grafted scaffold. To this end, there are a variety of particulate bone graft materials which can be effective scaffolds for new host bone formation.

Autogenous bone grafting is the gold standard in grafting procedures because it incorporates all three ideal characteristics of bone grafts; osteoconduction (space maintenance), osteoinduction (cell signaling with growth factors such as BMP's, PDGF, TGF-β and VEGF), and osteogenesis (contains osteogenic progenitor cells capable of laying down new bone matrix). However, it is also associated donor site morbidity and limited availability intraorally. To avoid such donor site morbidity, and to have unlimited availability of graft material, three classes of non-autogenous bone substitutes have been developed: allografts, xenografts, and alloplasts.

Alloplasts are synthetically developed bone substitutes that consist of some combination of hydroxyapatite, β-TCP, polymers and/or bioactive glasses. These formulations have traditionally demonstrated inferior long-term clinical outcomes and are frequently utilized in patients who for personal or religious reasons choose to forgo treatment with allografts or xenografts. [8]

The most common xenograft is bovine derived bone particulate which is a mixture of cortical and cancellous bovine bone that is deproteinized (DBBM) but leaving its trabecular

macrostructure maintained. The most advantageous feature of DBBM is that of all of the particulate graft materials, DBBM is least prone to dimensional change during healing and maturation and is therefore useful in contour augmentation procedures or sinus augmentations.

Allografts are harvested from human cadavers and are processed to suppress their antigenic potential and sterilized by a number of different proprietary treatments including radiation, freezing and chemical treatment. Some of these grafts then go through an additional step of demineralization in cold, diluted hydrochloric acid to expose the bone morphogenic proteins (BMP's) associated with the collagen fibrils. For this reason, allografts which are mineralized (FDBA) are considered osteoconductive only, while demineralized allografts (DFDBA) are considered both osteoconductive and osteoinductive due to the exposure of the BMP's associated with the exposed collagen fibrils during demineralization. [9] These graft materials are very versatile and can be used in a wide range or procedures in periodontal regeneration or implant dentistry from guided tissue regeneration to ridge and sinus augmentations. [10–12]

Particulate allografts function by maintaining space for angiogenesis, osteogenic cell migration and eventual bone formation by replacement of the allograft scaffold. Although space maintenance is essential for new bone formation, the resultant dead space is left without a blood supply, which compromises the immune response to the inevitable bacterial contamination of bone grafting procedures in the oral environment.[13] This problem is often exacerbated when soft tissue dehiscence's or membrane exposures provide an open communication between the oral environment and the grafted area during the postoperative period. When sinus augmentation is performed with vertical ridge augmentation, membrane exposures a membrane exposure rate of 12.5% has been reported.[14] A recent systematic review and meta-analysis reported the combined rate of these complications during ridge augmentation procedures including membrane exposure,

soft tissue dehiscence, and acute infection/abscess to be 16.8%.[15] Such bacterial contamination of the graft has been shown to retard bone formation[9,16] or otherwise necessitate partial or even complete graft removal.[17,18] In some cases, infection can even lead to resorption of basal bone [15] which in addition to scar tissue formation will leave the patient is an even more compromised condition than they were pre-operatively.

The lack of blood supply to the grafted area not only compromises the patients' immune response to such bacterial contamination in the immediate postoperative period, it also compromises the action of orally administered antibiotics; a common practice preoperatively, postoperatively or in response to a healing complication for such procedures.[18,19] While these systemic antibiotics can have some effect on infections surrounding the surgical site, only those antibiotics which are contained in the initial blood clot will be available within the surgical site until the reestablishment of blood supply.[20] This has prompted many clinicians to place antibiotic mixtures locally, directly into the surgical site at the time of surgery.[21–23] While higher concentrations of antibiotic are achieved with this technique,[22] the pharmacokinetics of such locally delivered drugs are insufficient to bridge the time gap between surgery and completion of angiogenesis into the dead space.

While orally administered amoxicillin is currently the antibiotic of choice for prevention of infection following oral surgical procedures due to its superior absorption, bioavailability and broad-spectrum coverage,[19] it is also associated a high allergic cutaneous reaction rate (5.14%),[24] as well as other side effects including headache, diarrhea, nausea, and vomiting.[25] Locally delivered antibiotics have many advantages over orally administered antibiotics: they are independent of patient compliance; do not require absorption in the gastrointestinal (GI) tract so are not associated with GI side effects; and they minimize the possibility of drug interactions,

allergy development, and antibiotic resistance by limiting exposure. Because locally administered antibiotics do not require absorption through the GI tract, antibiotics administered in this way can be selected based on their specific characteristics at the site of action. This is even more important given the recent guidelines on antibiotic stewardship.[26]

Cefazolin is a first generation cephalosporin that is effective against the most common pathogens associated with infection of allografts in oral surgical procedures (alpha-hemolytic streptococci and S. viridans)[27], making it an ideal candidate for locally administered antibiotic prophylaxis in dentoalveolar particulate bone grafting procedures.[28] In addition, unless the patient has a history of a true anaphylactic reaction to a penicillin antibiotic (0.015–0.004% of patients given a penicillin related drug)[29], it is safe to use in penicillin-allergic patients.[30] Cefazolin is often used in medicine for surgical prophylaxis,[31] with some authors investigating its use by local delivery showing prolonged surgical site concentrations above minimum inhibitory concentration when compared to intravenous administration.[22,32] Prolonged release of local drug delivery is important during bone grafting procedures because the locally delivered antibiotic should ideally remain active in the surgical site until angiogenesis of the site is adequate to reestablish host immune response.

Several studies have investigated local drug delivery in bone regeneration using natural or synthetic polymer scaffolds such as gelatin, chitosan, alginate, collagen, hyaluronic acid, poly(glycolic acid), poly(lactic acid), copolymers of poly(DL-lactic–glycolic acid) (PLGA), polycaprolactone (PCL) and many others. [33–37] The use of synthetic polymers has been found to be advantageous in their tunable properties such that the degradation rate, mechanical properties, and macroscopic shapes can be tailored to the specific application.[38] These synthetic polymers are especially useful as hydrogels, which are colloidal mixtures that exhibit viscoelasticity,

meaning they are capable of maintaining their volume and shape until agitated because of the simultaneous existence of both liquid (water) and solid (polymer) phases. Many hydrogels exist in nature, such as in cartilage or in the vitreous humor of the eye, while others are man-made for a variety of industrial and medical uses, such as glue, disposable diapers, contact lenses, breast implants or tissue engineering and drug delivery applications.

Recent research has shown the utility of hydrogels made by linking together highly branched macromolecules called dendrimers, which themselves have highly tunable properties which are advantageous for drug delivery applications. Dendrimers derive their name from the Greek word "dendra" meaning tree-like structure. These molecules have three components: a central core, repetitive branching units (the layers of which denote the generations (G) of the dendrimer), and terminal groups located on the periphery of the molecule which determine its reactivity. In general, the higher the generation of the dendrimer, the larger the molecule becomes, and the more end-group (termini) functionality it develops.

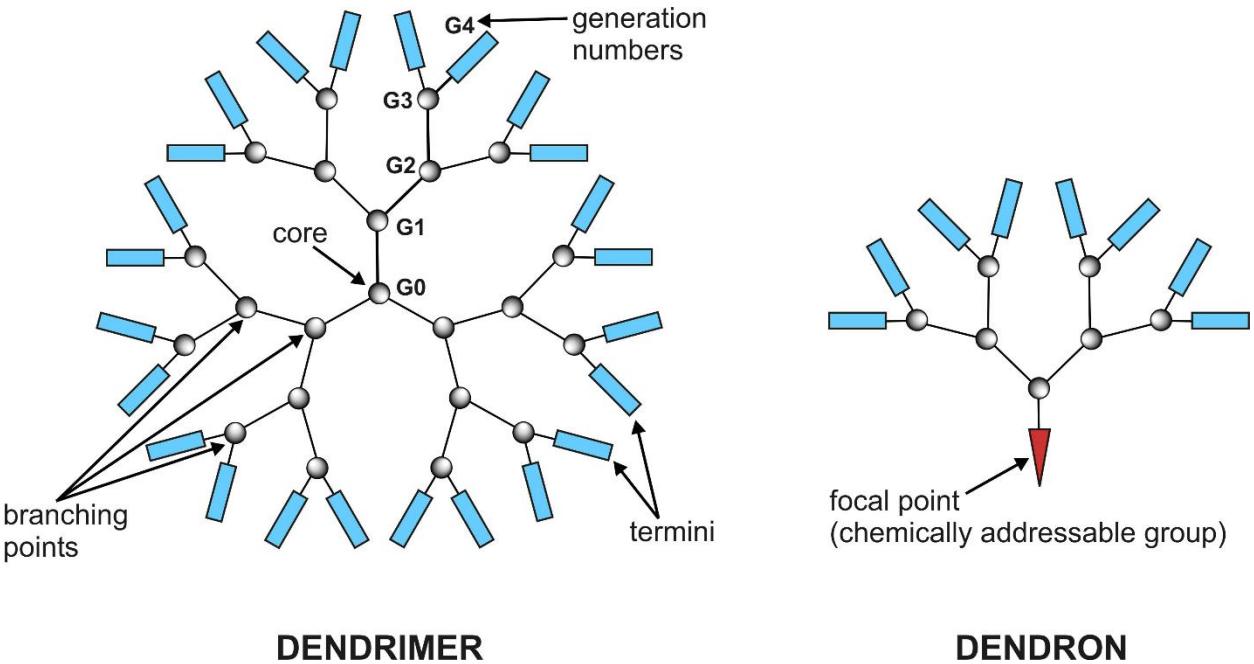

Figure 1: Shows the tree-like structure of dendrimers. The higher the generation (G) of the dendrimer, the higher molecular weight and the more end-group (termini) functionality it develops.

To control the release of locally administer cefazolin in alveolar bone grafting procedures, we propose using polyamidoamine (PAMAM) dendrimers to make dendrimer hydrogel (DH),[39] which have been heavily studied to construct nanoparticulate delivery systems.[40–44] The positively charged amine groups located on the periphery of PAMAM dendrimers make them amenable to crosslinking via *aza*-Michael addition with polyethylene glycol diacrylate (PEG-DA). This reaction is highly efficient and requires no catalyst or initiator and can occur at room temperature. PAMAM dendrimers crosslinked with PEG-DA are hydrophilic, degradable in vivo via pH-dependent aminolysis, and have shown low cytotoxicity given low concentrations and minimal free amine groups. [45] Such positively charged free amine groups can interact with the negatively charged cell membranes and effect their stability and periability.[46] With this in mind, a low cytotoxicity can predictably be achieved by limiting the concentration of and the number of free amine groups in the hydrogel. Both PAMAM and PEG-DA have been approved for human use by the FDA.

This new platform uniquely integrates the properties of *in situ* gelling, mucoadhesive polymers and nanoparticles (Fig. 2). DH possesses unique spatial structure and configuration with tunable physiochemical properties and high flexibility in delivering drugs of different types.[39,47–49]

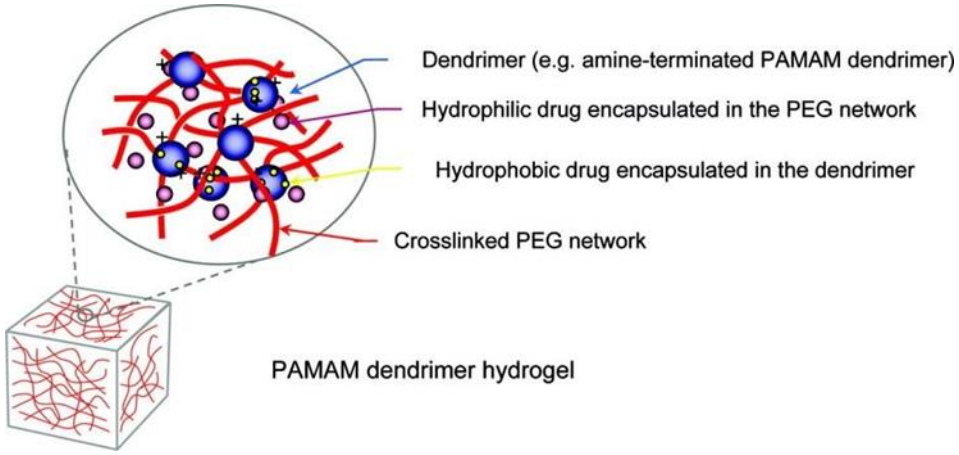

Figure 2: Schematic of a novel dendrimer hydrogel (DH) platform. Hydrophobic drugs can be encapsulated into hydrophobic dendrimer core, while hydrophilic drugs can be dispersed into the crosslinked PEG network.

This novel platform:

(i) facilitates the release of combined drugs at the ratio prescribed by confining the drugs into particulate structures; and

(ii) has high drug encapsulation capacity for both hydrophilic drugs (or drug salt form) and hydrophobic drugs and it enables programmable synchronized release of the delivered drugs.

In addition, our new scaffolds have the potential to engage bone regeneration simultaneously. Bone defects often have irregular shapes. In this scenario, in situ-forming or injectable scaffolds help fill irregular defects whereas preformed materials cannot.[50–56] We will generate cefazolin-loaded DH enmeshing particulate allografts to form an injectable formulation that is structurally adaptable to accommodate multifaceted needs including biocompatibility, mechanical strength, bone regeneration, and injectability. The aim of the present study is to utilize DH to prolong the release of cefazolin from particulate bone substitutes *in-vitro* as the first step towards utilizing DH in oral bone regeneration.

Methods

The experiments were designed to have 2 groups based on the presence of dendrimer hydrogel (DH): test group (containing DH) and control (no DH). Within each group, there were three subgroups based on the composition of the bone graft: DFDBA, FDBA or no bone graft. All tests were run in triplicate.

Cefazolin solution was prepared from powder (Sigma) dissolved in pH 7.4 PBS at room temperature to a final concentration of 5mg/mL. Test group solutions were prepared using EDA-core PAMAM dendrimer generation 5 (G5) purchased from Dendritech (Midland, MI, USA) at a concentration of 10 wt % G5 and 5mg/mL cefazolin. Allograft samples donated from LifeNet Health (50:50 c/c FDBA, 50:50 c/c DFDBA) were suspended in both test and control solutions at a volume of 0.1cc of allograft. Polyethylene glycol diacrylate (PEG-DA, Mn=575 g/mol) was then added to the test solutions in a 1:1 amine: acrylate molar ratio and immediately mixed using a Vortex Mixer at 3200 rpm and left to solidify overnight at room temperature on an orbital shaker at 100 rpm. After 24 hours, all groups were sealed in dialysis bags along with 7.4 pH PBS pre-heated to 37^{o}C for a total of 30mL of PBS in each group. Groups were then kept in a 37 ^{o}C bath for the duration of the experiment. Groups were inverted and 1mL aliquots were taken and replaced with 1mL pre-heated PBS at time points 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 12hrs, 24hrs, 48hrs, 72hrs. Aliquots were stored at 4 ^{o}C until analysis. The reverse-phase high performance liquid chromatography (RP-HPLC) system (Waters, MA, USA) consisting of a system Waters 1515 isocratic HPLC pump, a model Waters 717plus autosampler and a model Waters 2487 dual λ absorbance detector was used in this work. An XTerra particle-based RP-HPLC column (length 150 mm, particle size 5 μm, RP18) was purchased from Waters (MA, USA). The mobile phase consisted of ultrapure water and acetonitrile with 60:40 v/v, pH adjusted to 8, with drops of triethylamine. The flow rate was set to 1ml/min, using UV detection at 270nm. The solutions were filtered through a 0.22μm filter before being injected, and the mobile phase was degassed by ultrasonic bath before being used. The peak areas were integrated automatically, and the standard curve was developed using samples of powder-form cefazolin in PBS in variable

concentrations. A line of best fit was then created based on the standard curve and used to translate the concentrations of cefazolin in the aliquots with correction for sampling dilution.

Statistical Methods

A restricted cubic spline model was utilized to model the nonlinear Cefazolin concentration by time and test for differences based on presence of DH, FDBA and DFDBA. Following the methodology established by Harrel (REF), percentiles were used to estimate 4 knots in the concentration curves (5, 35, 65, 95th percentiles). Logistic growth models were used to estimate the maximum concentration, rate of increase, and the time at which the concentration reached 50% of the maximum for the final models. SAS EG v.8.2 with SAS v9.4 was used for all analyses (SAS Institute, Cary, NC). Significance level was preset at 0.05 level.

Results

All splines in the RCS model were statistically significant in estimating the nonlinear relationship between time and the cefazolin concentration (p-values<0.0001). There was also a significant effect of presence of DH (p-value<0.0001). The effect for DFDBA was not significantly different from no bone graft (p-value=0.4308), nor was the effect for FDBA as compared to no bone graft (0.3345). Results from the RCS model are presented in Figure 3.

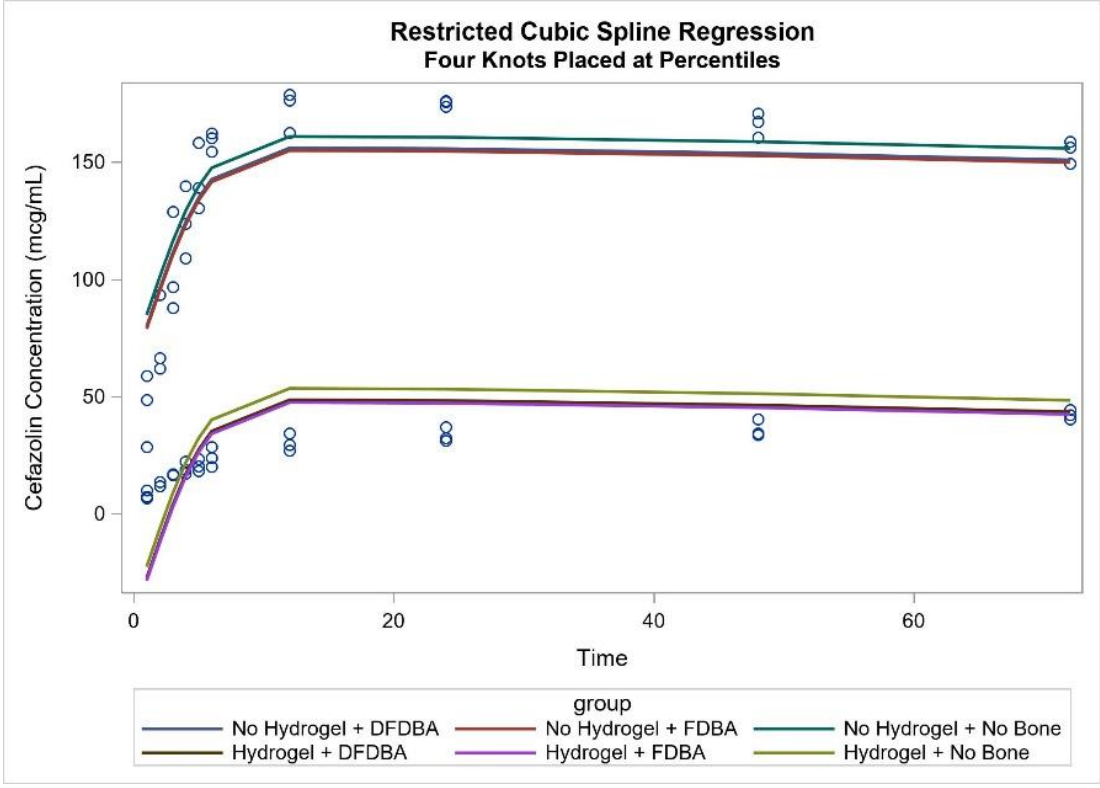

Figure 3: Restricted Cubic Spline Model for Cefazolin Concentration by Time, Bone Graft Material, and Presence of Hydrogel

Due to the differences between the concentration with and without DH, individual logistic growth models were fit for the two separately (Figure 4). For samples with DH (test), the estimated maximum concentration achieved was 36.97mcg/mL (95% CI: 34.58-39.36). The estimated growth rate is 0.27 (95% CI: 0.17-0.37). At 4.17 hours (95%: 3.26-5.07), the concentration reached half the maximum value. For samples without DH (control), the estimated maximum concentration achieved was 167.4mcg/mL (95% CI: 160.4-174.4). The estimated growth rate is 0.70 (95% CI: 0.54-0.87). At 2.36 hours (95% CI: 2.05-2.67), the concentration reached half the maximum value. These results demonstrate that with DH, the cefazolin concentration increases at a much slower and steady rate than controls (without DH).

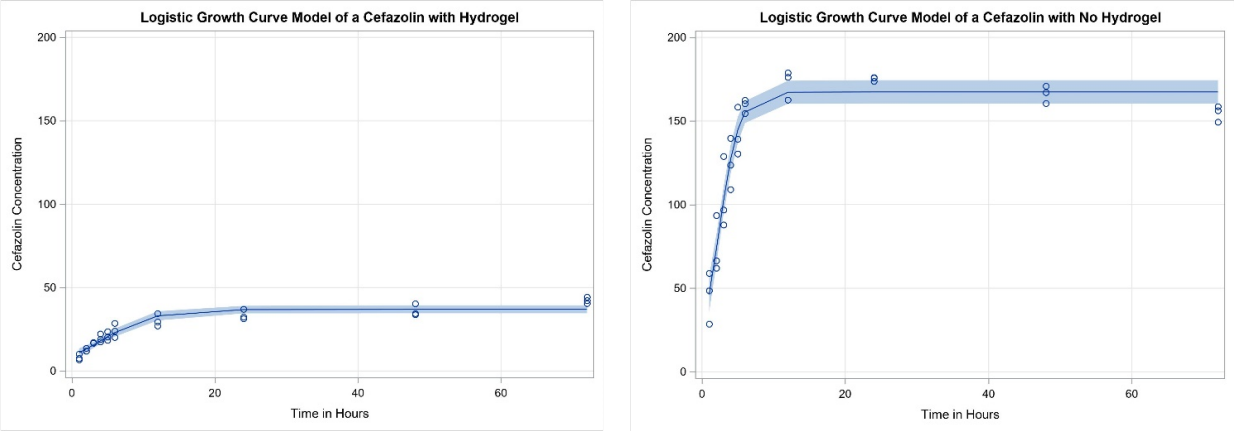

Figure 4: Logistic Growth Curves for Cefazolin Concentration with and without Hydrogel

Discussion

In this investigation, we were able to show the effectiveness of PAMAM/PEG-DA dendrimer hydrogels (DH) in slowing the release of cefazolin *in-vitro*. The mechanism of this effect could be due to one or multiple of the following interactions between cefazolin and the DH:

1. Entrapment: many drugs can become entrapped within the network of the crosslinked DH.
2. Electrostatic interactions: positively charged unreacted amine groups on the surface of the dendrimers could have interacted with the negatively charged cefazolin carboxyl groups.
3. Hydrogen bonding between the cefazolin and the hydrogel network.

There is a clear dichotomy between entrapment and electrostatic interactions in this scenario, as any free amine group that is used to react with PEG-DA to facilitate entrapment of the drug will negate an amine group that could participate in electrostatic interactions. However, as stated before, the number of free amine groups should be minimized to limit the cytotoxicity of the DH

so it would be prudent to favor entrapment and hydrogen bonding when possible as the drivers of cefazolin-DH interactions. No chemical reaction is completely efficient and hence there will always be some unreacted amine groups in the solution.

One unexpected result from this experiment was the finding that the control group with neither DH nor bone graft did not show a consistent solution concentration. This may have been due to the use of the dialysis bag in the experiment. The objective of the dialysis bag was to simplify the sample analysis by preventing large molecular weight DH degradation products from exiting the dialysis bag. As a byproduct however, this may have slowed the diffusion of cefazolin outside of the dialysis bag where it could be sampled. This suggests that all groups likely had a faster release profile than was observed in our experiment as a dialysis bag was used in all groups.

It was hypothesized that the presence of bone graft material would delay the release of cefazolin as cefazolin might bind to the mineralized portions of the graft. No evidence of such an interaction was found in this investigation, however this does not rule out the possibility that such an interaction exists given the small sample size.

One limitation of this study was that we did not test the cytotoxicity of the DH to osteoblasts. Although previous studies have examined the effect of DH on other human cell lines [43,48], it will be important to examine their effect on osteoblasts if they are intended to be used in bone grafting procedures to be sure that they do not impede bone formation.

Future experiments may focus on the cytotoxicity of the cefazolin/DH drug delivery system by cell culture with osteoblasts or *in-vivo* experiments. Alternatively, studies concerning the delayed

release of growth factors may be an interesting area of study as such therapies are currently receiving much attention.[57]

Conclusion

Prevention of infection in bone grafting procedures is important for successful treatment. Antibiotics delivered intraoperatively to the grafted site should ideally remain active until blood supply and host immune response is established. The results of this investigation that dendrimer hydrogels can effectively prolong the release of cefazolin *in-vitro*. This is but one example of the many potential uses of dendrimer hydrogels (DH) as a drug delivery system in periodontics and implants dentistry as they allow clinicians to customize drug release kinetics, mechanical properties, and in-situ gelling for specific clinical applications.

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