Dental Research Journal

Original Article

Drug release kinetics and biological properties of a novel local drug carrier system

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Received: 24-Jun-2020
Revised: 23-Apr-2021
Accepted: 22-May-2021
Published: 22-Nov-2021

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ABSTRACT

Background: The purpose of this *in vitro* study was to investigate drug release kinetics and cytotoxicity of a novel drug delivery system for treatment of periodontitis.

Materials and Methods: This *in vitro* study addresses the fabrication of a polycaprolactone/alginic acid-based polymeric film loaded with metronidazole, as a basic drug in the treatment of periodontal diseases. Films were prepared by solvent casting technique. Four formulations with different percentages of drug by weight (3%, 5%, 9%, and 13%) were prepared. Drug release kinetics were investigated using ultraviolet–visible spectroscopy during (one week). Data were analyzed using repeated measures ANOVA. Cytotoxicity of drug-loaded system extracts was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using L929 cells after 24-h incubation. The results were evaluated according to ISO standard 10993-5 and assessed using ANOVA and Tukey's tests at a significance level of $P < 0.05$.

Results: All polymeric films showed a burst drug release followed by a gradual release. Drug release data were fitted well with the first-order kinetic model in all drug-containing formulations indicating that drug release is a fraction of remaining drug in the matrix. Drug release is mainly driven by diffusion of medium into the composite matrix. 3%wt metronidazole-containing formulation exhibited the best MTT result.

Conclusion: The findings of this study supported the synthesis of drug-loaded periodontal films with 3% metronidazole due to better biological properties along with the ability of acceptable drug release to eradicate anaerobic periodontal bacteria.

Key Words: Drug delivery system, metronidazole, periodontal diseases, pharmacokinetics, toxicity

INTRODUCTION

Approximately 700 bacterial species have been recognized in the oral cavity of humans.[1,2] These bacteria produce an organized biofilm on the surfaces[3] and form the commensal biofilm members in persons who are periodontally healthy. In the case of poor oral hygiene, biofilm biomass increases, and situation becomes in favor of growth and reproduction of anaerobic species. The presence of bacteria promotes

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How to cite this article: Shafiei F, Ghavami-Lahiji M, Kashi TS, Najafi F. Drug release kinetics and biological properties of a novel local drug carrier system. Dent Res J 2021;18:94.

misdirection of host immune response and increased inflammatory response.[4,5]

In periodontal diseases, bacteria in the oral cavity shift from commensal to pathogenic bacterial flora and eventually lead to the destruction of tooth-supporting tissues.[4] The basic therapy for periodontal disease is mechanical debridement. Although mechanical debridement plays a key role in diminishing bacterial load, some microorganisms and endotoxins remain inaccessible in deep complex periodontal pockets and concavities. Failure in eradication of pathogenic microorganisms may lead to bacterial recolonization and treatment failure.[6,7]

Systemic administration of antibiotics appears to be effective in treatment of periodontal diseases. However, the potential risks of superimposed infections, formation of resistant strains, and gastrointestinal disorders caused by long-term consumption of systemic antibiotics cannot be ignored.[6,8,9] Nowadays, local drug delivery (LDD) systems have become the focus of interest in recent years to solve these problems and optimize periodontal therapy.

So far, several experimental and commercial LDD products have been introduced. However, there is uncertainty about sustainability of gel-based drug carriers[10,11] due to rapid elimination of their matrix.[12,13]

This *in vitro* study deals with fabrication of drug-loaded periodontal films using polymers that are biocompatible. Polycaprolactone (PCL) was selected as the main polymer in matrix due to its excellent mechanical properties and flexibility.[14,15] Metronidazole which is a basic drug in the treatment of periodontal diseases was used in the present study. Blending of PCL with alginic acid in this carrier system would enhance water penetration when loaded with a hydrophilic drug and helping to better drug release.[16] The effect of this LDD system on the complex of major periodontal pathogens was investigated in our previous study.[17]

The purpose of the present study was to investigate drug release kinetic and *in vitro* cytotoxicity of this novel drug-loaded polymeric film in order to apply as a LDD system suitable for access to the depth of the periodontal pockets.

MATERIALS AND METHODS

Formulation

In this *in vitro* study, a new PCL and alginic acid-based LDD system was designed for controlling residual bacteria after mechanical debridement. Metronidazole was purchased from Sigma-Aldrich (St. Louis, MO). Four formulations with different percentages of drug by weight (3%, 5%, 9%, and 13%) were prepared which were named M1 to M4. The details of synthesis and characterization of the polymeric films were explained in our previous study.[16] The polymeric films were sterilized using gamma rays with dose of 25 kGy and dose rate of 1.62 Gy/s (Gammacell GC-220 Irradiator).

Drug release

Preparation of standard curve (calibration curve)

One hundred milligrams of metronidazole was weighed and dissolved in 100 ml of phosphate-buffered saline (PBS) (pH 7.4) to yield a stock solution of 1 mg/ml. Ten milliliters of this stock solution was diluted to make up to a 100-ml volume to yield the concentration of 100 μg/ml. Substock solution was diluted to five concentrations in the range of 5–25 μg/ml by pipetting out 0.5, 1, 1.5, 2, and 2.5 ml from this solution and diluted to 10 ml to make 5, 10, 15, 20, and 25 μg/ml concentration of solutions.

The absorbance of the prepared concentrations was then measured using an ultraviolet–visible (UV–vis) spectrophotometer (Alpha-1860, Thomas Scientific, USA) at maximum wavelength (λ_{max}) of drug. The λ_{max} for metronidazole was 320 nm. Calibration curve was created by plotting the absorbance versus the known drug concentration data. The calibration curve was used to calculate the concentration of unknown specimen during the experiment.

In vitro evaluation of drug release from drug-loaded polymeric films

In order to measure drug release from the drug-loaded formulations, polymeric films with the dimensions of 1 cm × 1 cm with a sample weight of nearly 0.06 g[16] were placed in microtubes containing 5 ml of PBS at 37°C in a shaking incubator.[18,19] At predetermined time intervals (3, 6, 12, and 24 h and then every day up to 7 days), an aliquot of 3 ml was removed to measure drug concentration by UV–vis spectrophotometry. Identical volume of fresh medium was added to the tubes to maintain the sink conditions. Cumulative drug release data were calculated and

fitted to various release kinetic models such as zero order, first order, Higuchi's model, Hixson–Crowell, Hopfenberg, and Korsmeyer–Peppas.[20]

According to Siepmann and Peppas, when the Korsmeyer–Peppas exponent ("*n*" value) is <0.5 for slab (thin film) geometry, a Fickian diffusion takes place, whereas if the value is between 0.5 and 1, it indicates a non-Fickian release mechanism.[21] For other geometries, such as cylinder and sphere, $n = 0.45$ and $n = 0.43$ are characteristic for Fickian diffusion, respectively.[21,22]

Assessment of *in vitro* cytotoxicity of polymeric films

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to investigate the effect of polymeric film on cell viability. Fibroblast cells L929 (NCBI C161) were prepared from the Pasteur Institute of Iran (Tehran).

This assay relies on the reduction of MTT by the mitochondrial succinate dehydrogenase changing the yellow color of water-soluble tetrazolium to purple formazan crystals. The formazan product is analyzed spectrophotometrically after dissolution in dimethyl sulfoxide or isopropanol. Optical density (OD) of the spectra is directly proportional to the number of viable cells.

In order to investigate the toxicity of the samples and their effect on cell growth and proliferation, the extraction process was performed according to the ISO 10993-5 (tests for *in vitro* cytotoxicity), whereby 1 ml of the culture medium was added per 3 cm^2 surface area of each sterilized sample. After 24 h, the medium was removed and added to the cells after dilution. A specific amount of roswell park memorial institute medium (RPMI) was also considered as control.

10^4 fibroblast cells per well together with 100 µL of medium were cultured in 96-well plates and incubated for 24 h at 37°C until the cells stick to the plate floor. After ensuring the cell adhesion, the culture medium was removed as much as possible and 90 µl of serially diluted extracts (2x, 4x, and 8x) plus 10 µl of fetal bovine serum (FBS) was added to each new well, and the cells were exposed to these extracts for 24 h. Then, culture medium was removed and 100 µl of MTT at a concentration of 0.5 mg/ml was poured into each well and incubated for 4 h. MTT medium was taken out from the incubator, and isopropranolol solvent was added to dissolve its formazan crystals. The solution in each well was pipetted in 15 min. Finally, the absorbance of the MTT solution was read using an ELISA Microplate Reader (STAT FA × 2100, USA) at 570 nm and compared with control values. Cell viability and cytotoxicity are calculated from the following relationships:

$$\text{Cell viability}\,(\%) = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{control}}} \times 100$$

$$\text{Cytotoxicity}\,(\%) = 100 - \text{Cell viability}\,(\%)$$

Investigation of cell adhesion to the samples

For morphological observation, each of the sterilized polymeric films was placed in each well of a 24-well plate. Subsequently, 30,000 fibroblasts in a 100 µl of medium were seeded onto the surface of polymeric films and incubated for 4–5 h. After the cells were stuck to the sample surface, specified volume of culture medium containing 10% FBS was added to each well.

Culture medium was removed in 24 h, and the polymeric films were washed in PBS solution for 30 s. They were fixed using 3.5 vol% glutaraldehyde and kept for 2 h at 4°C. Subsequently, the fixator media was removed, and the specimens were washed with deionized water, dehydrated using ethanol (40%, 60%, 80%, and 100%), and then dried before sputtered with gold. The morphology of the adhered fibroblast cells was observed using field emission scanning electron microscopy (SEM).

RESULTS

Drug release

Standard curve (calibration curve)

The calibration curves of metronidazole in PBS are displayed in Figure 1.

In vitro evaluation of drug release from drug-loaded polymeric films

The release profiles of different formulations of the polymeric films during 1 week are represented in Figure 2. Cumulative drug release of metronidazole within 3 h was found to be 180 ± 5.65 µg/ml for M1. Increasing drug content resulted in higher drug release so that M2, M3, and M4 released 341.5 ± 32.81, 526.22 ± 63.91, and 829.11 ± 99.35

μg/ml, respectively. It was found that M1 formulation released approximately 40% of the drug content within 12 h. However, approximately 40% of the drug in M2 to M4 formulations was released within 3 h and reached the plateau after 4 days.

Investigation of drug release kinetics using mathematical models

The data obtained from the mathematical models are enlisted in Table 1. Drug release data were fitted well in the first-order kinetic model in metronidazole-containing formulations. First-order kinetic model can be successfully used to describe drug release from polymeric films indicating that drug release mechanism is concentration dependent. The amount of drug release decreases with decreasing concentration gradient over time. Different modeling graphs are provided in appendix. Our study revealed that n values for Korsmeyer–Peppas model in different formulations were within a range of 0.37–0.46 [Table 2].

Assessment of *in vitro* cytotoxicity of polymeric films

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

According to the findings, the cell viability on polymeric films was increased by the addition of the dilution factor [Figure 3]. It was especially more obvious for the cells treated using 4x and 8x diluted samples, and M1 presented the highest cell viability. There was no significant difference among metronidazole-containing groups in the diluted samples except for M1 sample with 2X dilution which showed a significantly higher OD value. Moreover, M3 with 2X dilution presented a significantly higher OD value compared to M4. There was no significant difference among metronidazole-containing groups in the diluted samples with 4X and 8X dilutions.

Investigation of cell adhesion to the samples

The SEM micrographs of the fibroblasts on the M3 specimen surface are provided in Figure 4. In 24 h of seeding, the fibroblasts on the surface of polymeric film were spindle-shaped, presenting fine cytoplasmic processes that seem to be keeping the cells attached to film substrate [Figure 4]. Thus, it has satisfactory cytocompatibility and has the potential to be used in drug carrier systems.

DISCUSSION

Oral cavity is considered as an open ecosystem where a dynamic balance is established between the entry of microorganisms, colonization methods, and host defense with the aim of eliminating them.[23] Periodontal infection is initiated by invasion of specific oral pathogens that colonize dental plaque

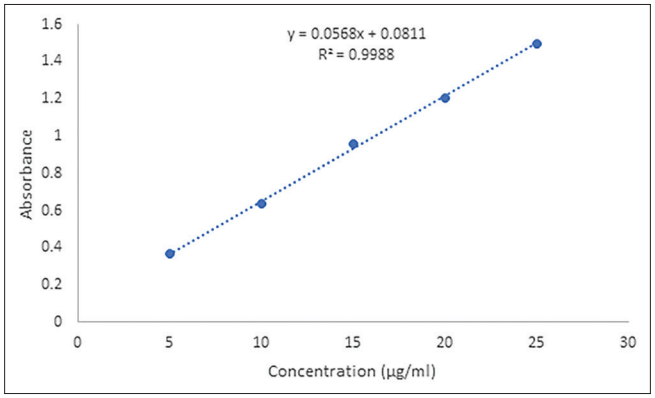

Figure 1: Calibration curve of metronidazole drug in phosphate-buffered saline.

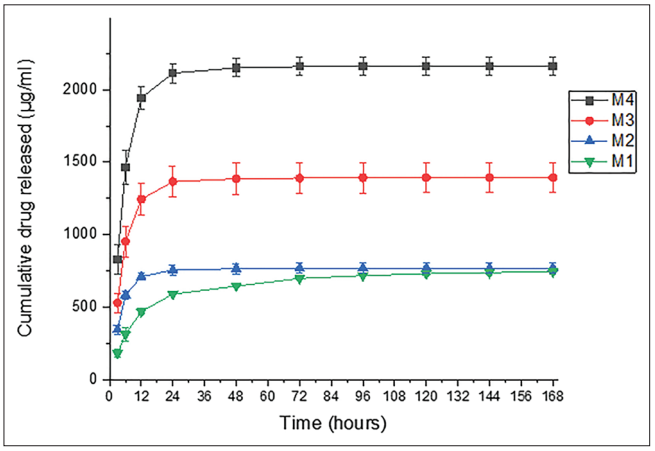

Figure 2: *In vitro* release of metronidazole during 1 week.

Table 1: R^2 value of drug release from different formulations

Formulations	Zero order	First order	Higuchi's model	Hixson-Crowell	Hopfenberg
M1	0.681	0.980	0.833	0.904	0.681
M2	0.40	0.971	0.567	0.749	0.40
M3	0.447	0.923	0.60	0.734	0.447
M4	0.449	0.974	0.66	0.787	0.449

M1=3%; M2=5%; M3=9%; and M4=13% metronidazole-loaded polymeric film

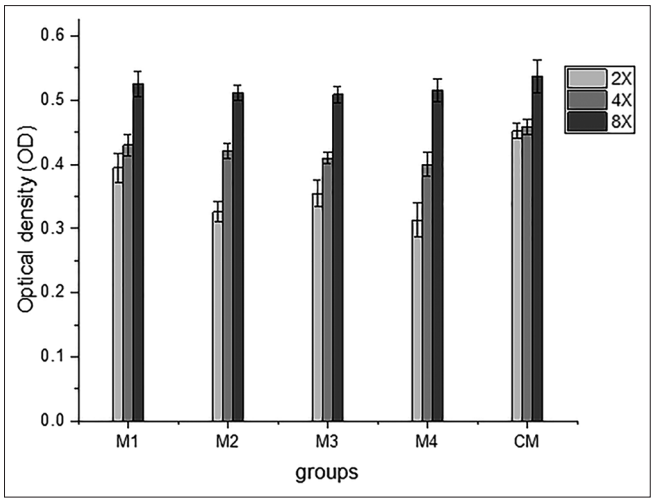

Figure 3: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay results of different dilutions of the polymeric film extract. (CM: Culture medium), (M1, 3%; M2, 5%; M3, 9%; and M4, 13% metronidazole-loaded polymeric film).

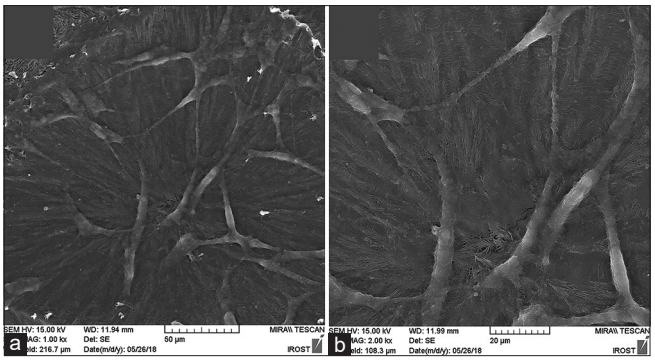

Figure 4: The scanning electron microscopy micrographs of the cell morphology on the surface of polymeric films at ×1000 (a) and ×2000 (b) magnifications.

Table 2: Goodness of fit and Korsmeyer-Peppas power-law release exponent

Formulations	r^2	Power law exponent (n)
M1	0.092	0.46
M2	0.854	0.37
M3	0.884	0.45
M4	0.889	0.44

M1=3%; M2=5%; M3=9%; and M4=13% metronidazole-loaded polymeric film

biofilms on tooth surface, and host immune response plays a central role in disease pathogenesis.[24] In the present study, PCL and alginic acid-based polymeric films were studied as a new drug carrier system. PCL, which is a Food and Drug Administration-approved biocompatible polymer, was selected as the main polymer in matrix due to its excellent mechanical properties, flexibility, and affordability. Alginic acid was incorporated, as the second hydrophilic antibacterial polymer to enhance water penetration, helping drug release. Metronidazole from the nitroimidazole family, which is one of the basic drugs in treatment of periodontitis, was used in the present study. We, in the previous study,[16] developed and characterized drug-loaded polymeric films, having acceptable physical properties, drug release kinetics, and antibacterial efficacy.

Metronidazole is a hydrophilic drug, first released by diffusion of dissolved drug molecules across the matrix. In this study, metronidazole-loaded polymeric films presented a high burst releasing followed by a relatively slow release until 4 days and then reached a plateau. The burst release phenomenon might be attributed to washing out of drug crystals at the surface of the films. If the matrix is occupied by more drug content, a faster release will be expected. Furthermore, it seems that increasing the amount of drug enhances hydrophilicity of matrix, encouraging water penetration, and consequently causing a faster drug release.

First-order kinetic model can be successfully used to describe drug release from polymeric films indicating that drug release mechanism is concentration dependent. Thus, drug release was a fraction of remaining drug in the matrix. The amount of drug release reduced with decreasing concentration gradient over time. The drug release was mainly driven by diffusion of medium into the composite matrix. However, other models such as zero-order model and Hopfenberg model were never able to describe drug release. It has been reported that transfer of solute (drug transport) from nondegradable polymer systems was mainly controlled by diffusion. These polymers can be made in two types of "reservoir" and "matrix" devices. In matrix-type devices, the release of drugs is more likely driven by a Fickian diffusion mechanism, which is influenced by the concentration gradient, distance, and swelling.[25]

Our study revealed that n values for Korsmeyer–Peppas model in different formulations were within a range of 0.24–0.46 [Table 2]. Thus, the release pattern mainly followed the Fickian diffusion-controlled mechanism. The findings were in agreement with Lan *et al.*,[26] Urmi *et al.*,[27] and Dhedage *et al.*[28] Regarding Tables 1 and 2, it could be concluded that the process of drug dissolution and water uptake would occur during the release of drugs from the prepared films. Whereas, surface erosion did not play a significant role. Penetration of water increases

molecular mobility of polymer systems. Mobility of polymer chains causes chain relaxation that enables drug release.

As reported in our first study,[16] tensile strength of metronidazole-incorporated polymeric films ranging from 5.80 ± 0.77 to 4.01 ± 0.24 MPa was significantly higher than those reported by similar studies.[18] Moreover, the highest elongation value was observed in M4, which is in agreement with the Couto *et al.*'s. study which reported a significant plasticizing effect of metronidazole drug on polymer.[18] Effectiveness of this drug-loaded system was also investigated in our second study against a periodontopathic multispecies model in terms of planktonic growth and biofilm formation assay. The findings of antibacterial tests against multispecies bacterial model were satisfactory in all groups and revealed more than 3 log CFU/mL reduction in pathogenic bacteria.[17]

We need a drug carrier system that presents initial burst release more than minimal inhibition concentration (MIC) of periodontopathogen bacteria, having good working properties; nevertheless, drug release must be lower than the range of cell toxicity. Formulations M2 to M4 released a high amount of drug. This unnecessary drug release, based on MIC of periodontal pathogens,[17] is also prone to cell toxicity hazards.[29] Although, it has been reported that MTT assay cannot adequately represent *in vivo* situation, because higher concentration of materials can be tolerated in an open system *(in vivo)* compared to a closed system *(in vitro)* due to the role of dynamic environment and presence of buffering agents existed in human body. It should also be taken into account that the gingival groove is a dynamic environment and the amount and flow rate of gingival crevicular fluid are correlated with the development of gingival inflammation.[30]

In the study conducted by Ferreira *et al.*, metronidazole preserved the highest fibroblast cell viability in comparison with other antibiotics. Although, higher concentrations of metronidazole caused disorganized fibroblasts.[29] Polymeric films loaded with 3%wt metronidazole exhibited the best MTT result in our study. Viable spindle-shaped fibroblasts in SEM photomicrograph confirm cytocompatibility of this drug carrier system. The least amount of this drug in the formulation is preferred for clinical use. This study showed the potential of drug carriers as a promising controlled release system, which can be employed as an adjunctive therapy after mechanical debridement.

CONCLUSION

Based on the results of this study, it can be concluded that drug release mechanism in drug-loaded polymeric films is concentration dependent. Drug release is mainly driven by diffusion of medium into the composite matrix. This approach can be considered as a beneficial way to get rid of periodontal pathogens without the side effects of systemic antibiotic. Polymeric films loaded with 3%wt metronidazole, compared to higher concentrations, revealed the higher cell viability.

Financial support and sponsorship

This study was supported by grant number 96-04-69-36754 from the School of Dentistry, Tehran University of Medical Sciences.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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