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Research Article

An Investigation and Characterization on Alginate Hydogel Dressing Loaded with Metronidazole Prepared by Combined Inotropic Gelation and Freeze-Thawing Cycles for Controlled Release

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Abstract. The purpose of this study was to investigate the effect of combined Ca^{2+} cross-linking and freeze-thawing cycle method on metronidazole (model drug) drug release and prepare a wound film dressing with improved swelling property. The hydrogel films were prepared with sodium alginate (SA) using the freeze-thawing method alone or in combination with ionotropic gelation with $CaCl_2$. The gel properties such as morphology, swelling, film thickness, and content uniformity and *in vitro* dissolution profiles using Franz diffusion cell were investigated. The cross-linking process was confirmed by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. *In vitro* protein adsorption test, *in vivo* wound-healing test, and histopathology were also performed. The hydrogel (F2) composed of 6% sodium alginate and 1% metronidazole prepared by combined Ca^{2+} cross-linking and freeze-thawing cycles showed good swelling. This will help to provide moist environment at the wound site. With the *in vivo* wound-healing and histological studies, F2 was found to improve the wound-healing effect compared with the hydrogel without the drug, and the conventional product.

KEY WORDS: alginate; Ca^{2+} cross-linking; freeze-thawing; swelling; wound dressing.

INTRODUCTION

Wounds include cuts, scrapes, scratches, and punctured skin. Colonization of open wounds with microorganisms may cause malodor, exudation, and even delay in wound healing; therefore, topical antimicrobial therapy is essential. The U.S. Department of Health (1) recommends the use an antibiotic ointment to prevent wound infection. Topical antimicrobial therapy offers advantages such as providing high and sustained concentration of medication at the site of injury (2,3). The use of topical antimicrobials is recommended when it is suspected that a wound is progressing towards overt infection or an interruption in the healing process is observed (4). This occurs before infection when a wound is failing to heal but the clinical signs of infection are not apparent, such as when the wound is critically colonized (5). Application of topical antimicrobial agents at this stage is recommended to redress the host-bacterial imbalance in favor of the host (5).

Traditionally, antimicrobials are available as uncontrolled release systems. Alternatively, some of them have been incorporated into the dressings to allow controlled release of the drug at the wound surface. Ideal wound dressing should maintain a moist environment around the wound and absorb the exudates which have been shown to fasten the reepithelialization rate (2,6,7). Cross-linked hydrophilic polymers such as alginates with high intrinsic water content can provide a moist environment by absorbing exudates (8–10).

Alginate is a hydrophilic, biocompatible, biodegradable, and relatively economical polymer (11). It has various medical applications as wound dressing, dental impression, and in cell culture. In one randomized controlled study by Läuchli's team (12) on the use of calcium alginate *versus* polyurethane film dressing for wound management in skin graft donors, the alginate dressing required fewer dressing changes and less leakage of exudates. Alginate hydrogels were prepared physically by repeated freeze and thawing (13) or chemically *via* ionotropic gelation using polyelectrolytes (14). Combined Ca^{2+} cross-linking and freeze-thawing cycles had been reported by Hua *et al.* (15) for controlled release of diclofenac sodium in the form of hydrogel beads. The process showed improved swelling behaviors and slowed the release of drug from the dual cross-linked beads (15).

Alginates were reported to dissolve at higher pH values than an acidic medium (16). As the pH environment of wounds had been recorded to be within the range of 7.15–8.9 (17), it can be considered as a convenient medium for the formulation of alginate dressings with controlled drug release (11).

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Other types of modern dressings containing antimicrobials such as films and wafers were reported as potential delivery systems for wound healing. One team assessed the healing effects of polyethylene oxide (Polyox)- and carrageenan-based solvent cast films loaded with streptomycin (30% *w*/*w*) and diclofenac (10% *w*/*w*) in chronic wounds (18). The films showed higher zones of inhibition against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* compared to the individual drug zones of inhibition. Lyophilized wafer loaded with antimicrobials reported to have the potential to reduce bacterial infection (19,20). Pawar *et al*. (19) used sodium alginate with Polyox (50/50) in the formulation of wafer dressings.

Metronidazole is a synthetic antibiotic and belongs to the 5-nitroimidazole group, which is highly effective against anaerobic bacteria and protozoa. Its mode of action is *via* the reduction of its nitro group which leads to the production of short-lived cytotoxic intermediates. The toxicity of the intermediates is due to their interaction with deoxyribonucleic acid and possibly with other macromolecules which results in and inhibition of nucleic acid synthesis. It is the only antimicrobial agent which can be used systemically and/or topically to treat wound infections (3, 21) as it reduces the malodor of anaerobically colonized wounds. Also, it has been reported in two studies that topical metronidazole increases the epithelialization during wound healing by secondary intention (22, 23) in rats. However, both studies used gel preparations for metronidazole.

There are no reports on using freeze-thawing and/or ionotropic gelation methods to prepare a controlled release metronidazole wound dressing; thus, the aim of this study is to develop metronidazole-loaded alginate wound dressings with controlled drug release and improved swelling behavior prepared by these methods and then to investigate *in vivo* its role on improving the process of wound healing.

MATERIALS AND METHODS

Materials

Metronidazole was given as a gift from Gulf Pharmaceutical Industries (UAE). Sodium alginate (SA) was purchased from Avonchem (UK). Human serum albumin (HSA) (mol. wt.= 66 kDa, albumin 97.31%); dihydrogen sodium orthophosphate, disodium hydrogen orthophosphate, and phosphate-buffered saline (PBS) tablets (pH 7.4); and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (UK). All other chemicals were of analytical grade.

Ten male Sprague-Dawley rats weighting 350–400 g were supplied by Dubai Pharmacy College and used to evaluate the *in vivo* wound-healing effect and histopathology of hydrogels. All animal care and procedures were conducted according to the Guiding Principles in the Use of Animals in Toxicology, as adopted in 1989, revised in 1999, and amended in 2008 by the Society of Toxicology (24). The protocols for the animal studies were approved by the Ethics and Research Committee at Dubai Pharmacy College. The rats were allowed free access to food and water at a temperature of 20–23°C and a relative humidity of 50±5% for 24 h prior to the experiments.

Preparation of Metronidazole Hydrogels by Freeze-Thawing (FT) Cycle

Hydrogels were obtained by freeze-thawing (FT) cycle. The pre-formulation studies began with solutions containing SA were prepared in deionized water with different proportions of SA (3 and 6% *w*/*w*). Metronidazole (1% *w*/*w*) was dispersed in SA solutions and mixed by vortexing for 1 h. The mixture was poured into a Petri dish, followed by freezing at −20°C for 18 h and thawing at room temperature for 6 h, for three consecutive cycles. The films were dried at 37°C for 24 h. The dose of metronidazole in the dressing was 10.5 mg/cm^2. Table I shows the formulation composition of films selected for further investigation.

Preparation of Metronidazole Hydrogels by Freeze-Thawing Cycle and $CaCl_2$ Cross-Linking (FT-$CaCl_2$)

Metronidazole (1% *w*/*w*) was dispersed in 3 and 6% *w*/*w* SA solutions under shaking in a water bath at 40°C until a homogeneous solution was obtained. The solution was dropped into a Petri dish containing a sufficient volume of a 5% *w*/*w* calcium chloride aqueous solution. The resulting alginate hydrogels were separated from the solution and washed several times with distilled water. The hydrogels were then subjected to a freeze-thawing cycle as described in the previous section. The films were dried at 37°C for 24 h.

Film Thickness

Film thickness was measured at three different points of the film using a manual micrometer (Mitutoyo Co., China), and the mean values were calculated.

Determination of Hydrogel pH

One gram from each formulation and the reference conventional product was accurately weighed and dispersed in 10 mL of purified water. The pH of the dispersions was measured with a pH meter (Hanna Instruments, HI8417, UK).

Swelling Ratio

Hydrogel samples were cut into 2-cm×2-cm pieces and dried at 50°C in an oven for 1 h and their dry weights (W_d) were immediately measured; then, they were soaked in distilled water and maintained at 37°C and their weights (W_s) were determined at specific time points, and the swelling ratio (SR) was calculated using the following formula:

$$SR\% = [W_s - W_d / W_a] \times 100 \quad (1)$$

Adsorption of Protein onto Hydrogel Surface

Pieces of hydrogel membrane of 2 cm×2 cm were immersed in 4 mL of PBS (pH 7.4) containing human serum

Table I. Composition of Metronidazole Hydrogel Wound Dressing Films

Formulations	Metronidazole (% *w*/*w*)	SA (% *w*/*w*)	5% $CaCl_2$ (mL)	Method of preparation
F1	1	3	–	F-T
F2	1	3	QS[a]	F-T/$CaCl_2$
F3	1	6	QS[a]	F-T/$CaCl_2$
F blank	–	3	QS[a]	F-T/$CaCl_2$

[a] The volume of 5% $CaCl_2$ used of for cross-linking is 15 mL

albumin at 37°C and shaken at 100 rpm for 24 h. Samples were then gently taken out and rinsed five times with PBS and placed in three wells containing aqueous solution of 1% sodium lauryl sulfate and shaken for 1 h at room temperature to remove the protein adsorbed on the surface.

The protein contents of each sample were measured by taking the difference between the protein concentrations before and after immersing the hydrogel pieces in the protein/phosphate buffer solution using an albumin reagent kit and reading the absorbance in an ultraviolet spectrometer at 280 nm.

In Vitro Drug Release from Hydrogels

Before *in vitro* drug release studies, the drug loading (assayed content) and uniformity of metronidazole within the films were determined by cutting the films into small pieces which were accurately weighed to 5 mg and hydrated in 10 mL of distilled water at 37°C with stirring and left overnight to completely dissolve. The concentration of metronidazole in distilled water was assayed by HPLC as shown below.

A synthetic hydrophilic membrane was mounted on a Franz diffusion cell (Copley Scientific, UK). The receptor compartment contained 22 mL of PBS. A piece (2 cm×2 cm) of the test formulation was applied to the membrane over an area of 2.543 cm^2 across the donor compartment. The donor cell was exposed to ambient temperature and covered with a plastic cover to prevent evaporation. The temperature of the receptor compartment was maintained at 37±0.5°C while the dissolution medium was stirred continuously with a magnetic bar. Samples were withdrawn from the release medium at 0, 0.5, 1, 2, 3, 4, 6, 7, and 8 h and replaced with an equal volume of PBS to maintain sink conditions. The samples were analyzed by HPLC. All experiments were conducted in triplicates.

Concentration of metronidazole within the film dressings as well as drug release in dissolution studies were analyzed using Agilent 1200 HPLC equipped with an autosampler (Agilent Technologies, Cheshire, UK) with a ChemStation® software program. The stationary phase used for analysis was a column packed with a C18 reverses-phase material (5-μm Hichrom ODS column) (Hichrom Ltd., Berkshire, UK). The mobile phase consisted of a mixture of methanol and potassium dihydrogen phosphate (1.36 g/L) in the ratio of 30:70 (*v*/*v*). The flow rate of the mobile phase was maintained at 1.0 mL/min, and detector wavelength was set at 315 nm (25). During each run, 10-μL volumes were injected.

Data Analysis of Drug Release

To analyze the *in vitro* release data, various kinetic models were used to describe the release kinetics:

1. The zero-order rate (Eq. 1) describes the system where the drug release rate is concentration independent:

$$C = k_o t \tag{2}$$

where k_o is the zero-order rate constant expressed in units of concentration/time and t is the time.

2. The first-order rate (Eq. 2) describes the release from the system where the release rate is concentration dependent:

$$\ln Ct = \ln C_o - kt \tag{3}$$

where C_o is the initial concentration of drug and k is the first-order constant.

3. On the other hand, Higuchi described the release of drugs from insoluble matrix as a square root of a time-dependent process based on Fickian diffusion (Eq. 3):

$$Q = kt^{1/2} \tag{4}$$

where k is the constant reflecting the design variables of the system.

Korsmeyer *et al.* (26) derived a simple relationship which described drug release from a polymeric system. To find out the mechanism of drug release, first 60% drug release data was fitted in the Korsmeyer-Peppas model:

$$Mt/M\infty = Kt^n$$

where $Mt/M\infty$ is the fraction of drug released at time t, K is the rate constant, and n is the release exponent. The n value is used to characterize different release mechanisms.

Thermal Analysis of Hydrogels with Differential Scanning Calorimetry (DSC)

Samples of pure metronidazole, sodium alginate, physical mixture of metronidazole-sodium alginate, and composite films were weighed (1–2 mg) into an aluminum pan, covered with an aluminum lid, and crimped into position. The pan was placed in an oven together with a blank (prepared exactly in the same way but without the sample). The samples and blank were continuously purged with nitrogen gas, and thermograms were recorded over a temperature range of 20–300°C with a programmed heating rate of 10°C/min and tested in triplicate and analyzed by DSC 8500 (PerkinElmer, Inc., Waltham, MA).

Fourier Transform Infrared (FTIR) Spectroscopy Evaluation

Infrared spectra of the samples of pure metronidazole, sodium alginate, physical mixture of metronidazole-sodium

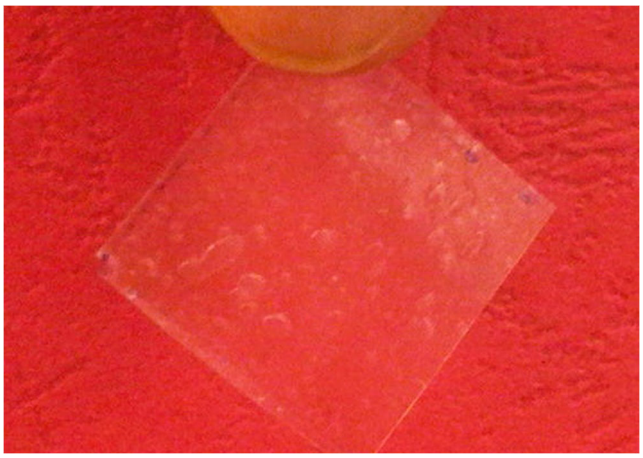

Fig. 1. The appearance of metronidazole hydrogel dressing (F3)

alginate, and composite films were obtained, using Cary 630 FTIR spectrophotometer with attenuated total reflectance sampling interface (Agilent Technologies, USA), in the frequency range of 4,000–650 cm^{-1} at 4-cm^{-1} resolution. The technique utilized a very small amount of each sample which was directly loaded into the system. The Cary 630 MicroLab PC software was used for data collection, and the Agilent Resolution Pro software used to determine peak positions.

In Vivo Wound-Healing Test

Male Sprague-Dawley rats weighting approximately 350–400 g were used to evaluate wound-healing characteristics of hydrogels. The dorsal hair of rats was shaved, and the animals were anesthetized with diethyl ether vapor. Two full-thickness skin wounds of 1.5-cm×1.5-cm area were prepared by excising the dorsum of rats and disinfected using 70% ethanol. The animals were divided into three groups (n=3) to receive different treatments. The excised wounds (group A) were treated with the SA wound dressings (F2) (2 cm×2 cm) with control wound (no treatment), those of group B were treated with the reference conventional product (1% metronidazole gel, Domina Pharmaceuticals, Syria) with control wound (no treatment), and those of group C were treated with blank formula (F2 without drug) with control wound (no treatment). After the experiment, rats were sacrificed by excess diethyl ether on 5 and 10 days after surgery. The wounds were grossly examined and photographed for characteristic evaluation.

For histopathological study, the skins including the entire wound with adjacent normal skin were excised and fixed in 10% buffered formalin. The specimen included the dermis and the subcutaneous tissue. Excised wound sites fixed in formalin were processed and embedded in paraffin, and sections of 3–5 mm were stained with hematoxylin and eosin. The histological profiles of individual rat skin and healing patterns of wounds were evaluated and photographed under a light microscope.

Table II. Thickness, pH, and Drug Content of Metronidazole-SA Dressings (n=3, ±SD)

Formulations	Thickness (mm)	pH	Drug content (%)
F1	0.14±0.01	7.66±0.01	99.1±1.2
F2	0.23±0.01	7.91±0.01	99.4±0.9
F3	0.24±0.11	7.91±0.01	99.5±2.3
F blank	0.91±0.13	7.32±0.01	–

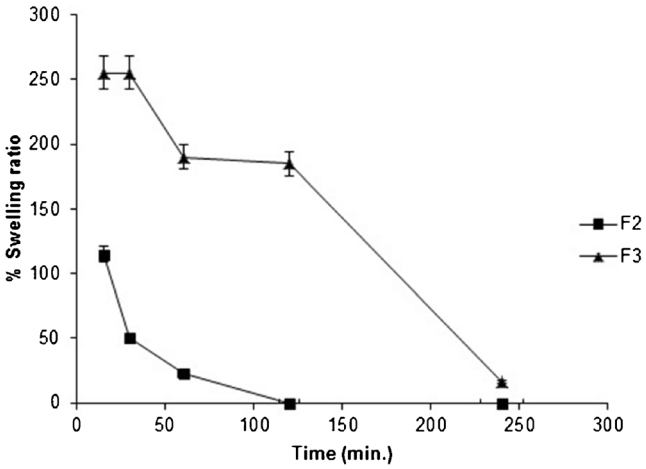

Fig. 2. Swelling ratios of metronidazole loaded alginate hydrogels *versus* time for F2 and F3 (n=3, ±SD)

Statistical Analysis

One-way ANOVA and independent-samples T test were applied if the variances in the groups are equal. If the variances are significantly different, Mann-Whitney test was used. Results are statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Formulation Development and Film Morphology

The films were generally transparent due to the miscibility in the aqueous media before film casting and easily removed from the Petri dish (Fig. 1). However, formulas prepared by FT-$CaCl_2$ method showed a "scaly" surface. This may be attributed to the double cross-linking of the dressings by FT and ionotropic gelation. The drug content

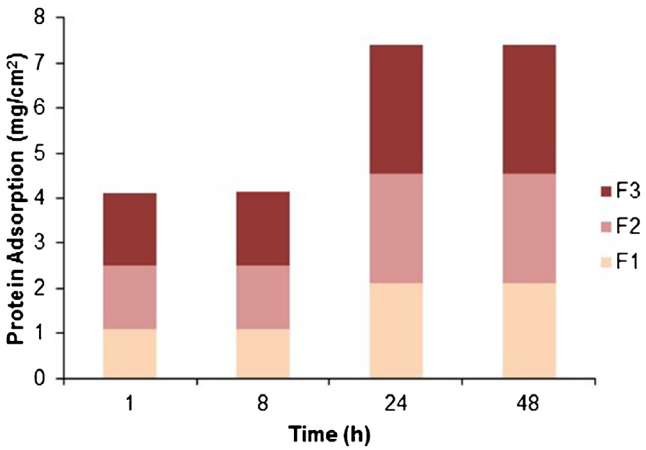

Fig. 3. Effect of SA concentration and method of hydrogel preparation on protein adsorption

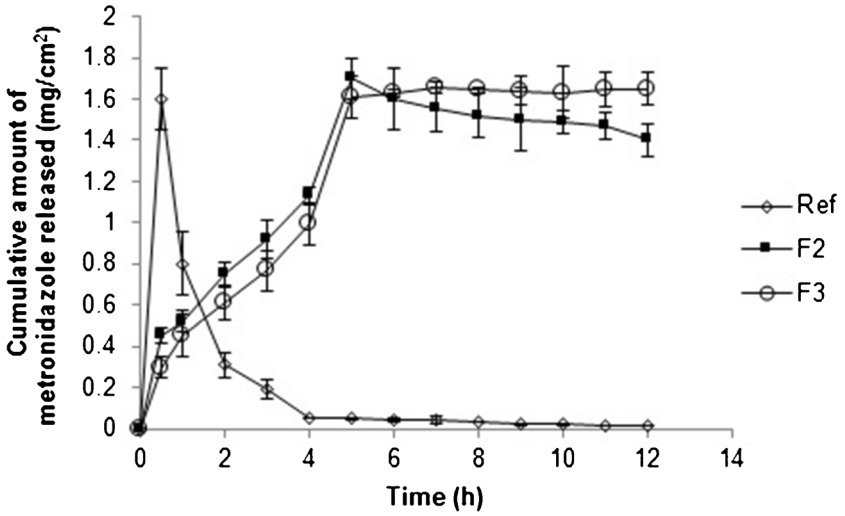

Fig. 4. Cumulative amount of metronidazole released from the gel and SA films against time (n=3, ±SD)

percentage for all formulas was within the accepted limits (>99%) as shown in Table II.

Films Thickness

The thickness of the prepared hydrogels was measured by the manual micrometer, and results are presented in Table II. The thickness of films has a significant effect on the film swelling property as it determines the concentration and depth of penetration of solvent molecules into the film (27), and subsequently, it affects the rate of drug release. Results showed that the thickness of dressings increased significantly (p<0.05) with the increase of SA concentration. Also, the incorporation of metronidazole obviously increased the formulas thickness. However, the method of cross-linking had a negligible effect on the dressing thickness since a minor difference (p>0.05) was observed in the thickness between F2 and F3.

Measurement of Film pH

The pH the prepared formula was adjusted close to the physiological pH of the normal skin in healthy people to avoid any irritation as well to optimize drug stability. Results are represented in Table II. Also, the physiological pH is important for the sustained release from the alginate film (16). At pH close to the physiological pH, the carboxylic groups of alginate will ionize and become $-COO^-$ in form. This event results in weakening the hydrogen bonding in the polymer chain and electrostatic repulsion from $-COO^-$ and higher swelling capacity (28).

Swelling Ratio

The swelling property of hydrogel dressing is a critical characteristic since it determines the degree of dressing bioadhesion to tissues and its capability to absorb the exudates and for uniform and prolonged release of the drug (29).

Figure 2 shows the swelling kinetics of the prepared hydrogel dressings with respect to time. It can be seen that the maximum swelling ability increased with increased SA proportion in the film. The formulation containing SA 3% *w*/*w* (F1) dissolved in the medium of PBS at pH 7.3 directly within few seconds with almost zero retention capacity. For F2 (SA 3% *w*/*w*), initially, the swelling rate was very high, and the water could be absorbed easily into the hydrogel, after which swelling decreased with time due to the loss of SA because of its solubility. Optimum swelling behavior was obtained with F3 (SA 6% *w*/*w*) which was prepared by FT and $CaCl_2$ cross-linking. F3 showed high absorption properties and also retained water for a longer period of time. This can be a potential candidate in highly exuding wounds where high water retention capacity is required for the dressing applied. The same behavior was reported by Kim *et al.* (30) for sodium alginate films.

Adsorption of Protein onto Hydrogel Surface

Protein adsorption and platelet adhesion are two important biological processes during wound healing (31). Protein adsorption is known to influence cell adhesion by adsorption of key adhesion molecules like fibronectin or vitronectin

Table III. Permeation Rate Constants and Coefficient of Determination (r^2) of Metronidazole from SA Wound Dressing

	Zero-order kinetics (μg h^{-1})		First-order kinetics (h^{-1})		Higuchi model ($h^{-1/2}$)		Korsmeyer's model (h^{-n})		
Formulas	K_0	r^2	K_1	r^2	K_H	r^2	K_{KP}	r^2	n
F2	0.1061	0.6521	0.0117	0.6514	0.5119	0.8079	1.755	0.9326	0.541
F3	0.2172	0.9278	0.236	0.9179	0.6662	0.9882	2.551	0.9955	0.438

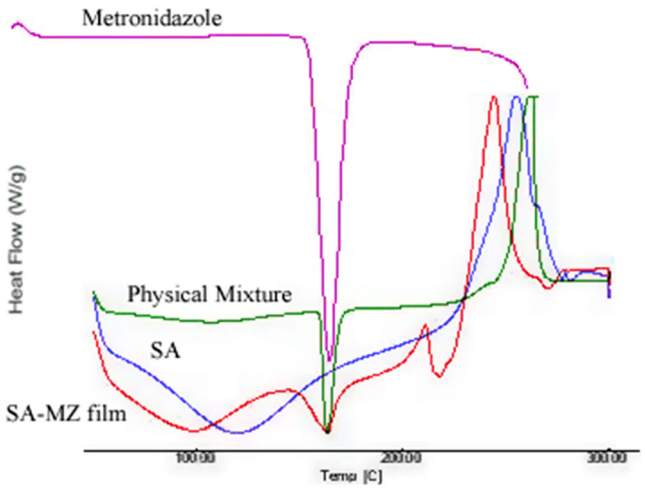

Fig. 5. Comparison of the DSC profiles of metronidazole, sodium alginate, and physical mixture of drug-polymer, and drug-loaded film

(32,33). In this work, the albumin adsorption was assessed for the adhesion of platelets to artificial surfaces. The higher the albumin adsorption, the lower was the number of adhering platelets. Figure 3 represents the HSA adsorptions onto the SA hydrogels. Results showed that the adsorption of HSA was not changed as the amounts of SA in the hydrogels increased, suggesting that SA does not influence the adhesion of platelets. Nevertheless, the method of dressing preparation had a significant ($p<0.05$) effect on HSA adsorption and maximum value was obtained with the formulation prepared by FT and $CaCl_2$ (F3). Also, protein adsorption onto the surface of the prepared hydrogels was significantly higher ($p<0.05$) after 48 h of the test, owing to the longer time for the protein-hydrogel surface interaction.

In Vitro Drug Release from the Films

To evaluate whether sodium alginate affected the release rates of metronidazole, the release studies on various metronidazole-loaded hydrogels were carried out (Fig. 4). The release rate of metronidazole in the hydrogel with 3% SA showed a "burst effect" compared with 6% SA hydrogel (total amount released was 1.7 mg/cm^2 in 5 h). This may be attributed to the complete dissolution of SA within the medium.

Furthermore, a controlled release profile was obtained from F3 dressing which released 1.64 mg/cm^2 mg of metronidazole in sustained manner up to 12 h. This indicates that SA concentration as well as the method of preparation has a significant impact on the release profile of metronidazole. Upon dissolution, the films were hydrated and characterized by gel formation which was not completely dissolved even after the dissolution period.

This may have a profound effect in terms of the wound-healing process. For chronic wound especially highly exuding wounds, they require more frequent changes for film dressings to avoid the collection of excessive amounts of exudate underneath the dressing which can cause skin maceration and damage to newly formed tissue (6). Thus, this sort of release profile may help to provide enough antibiotic to reduce the bacterial load before the next change of wound dressing.

Data Analysis of Drug Release

To study the release data from the tested formulations, the following plots were made with the coefficient of determination (r^2):

1. Cumulative drug released *versus* time (zero-order kinetic model).

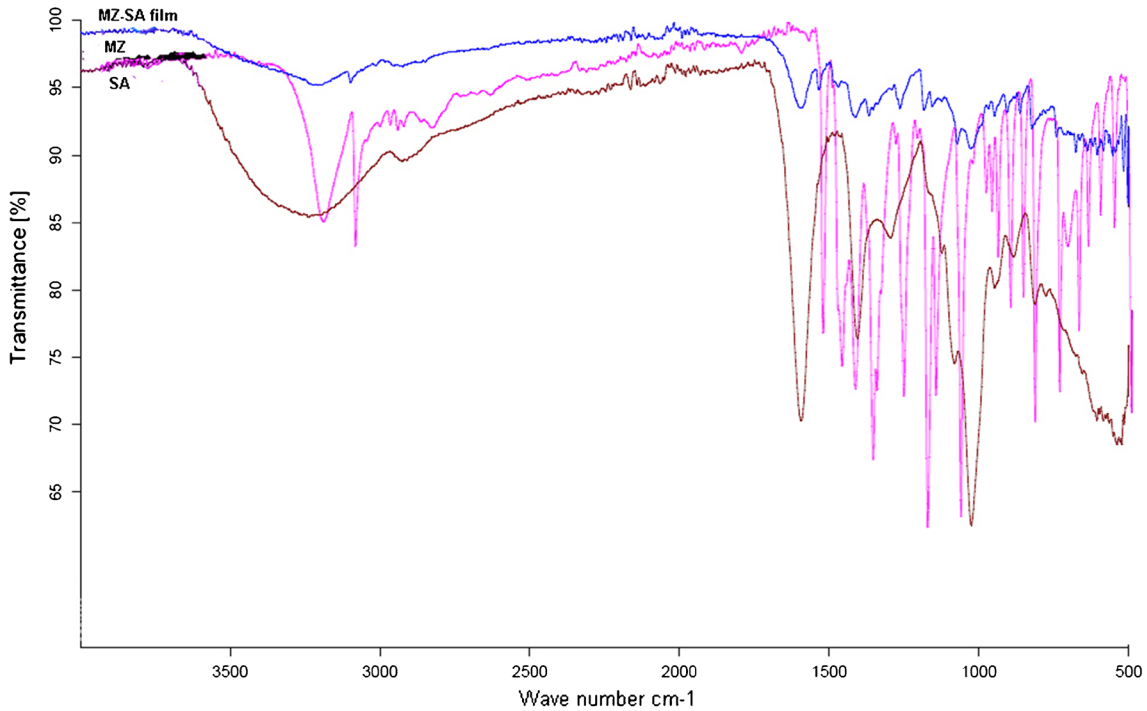

Fig. 6. FTIR spectra of metronidazole, sodium alginate, and drug-loaded film

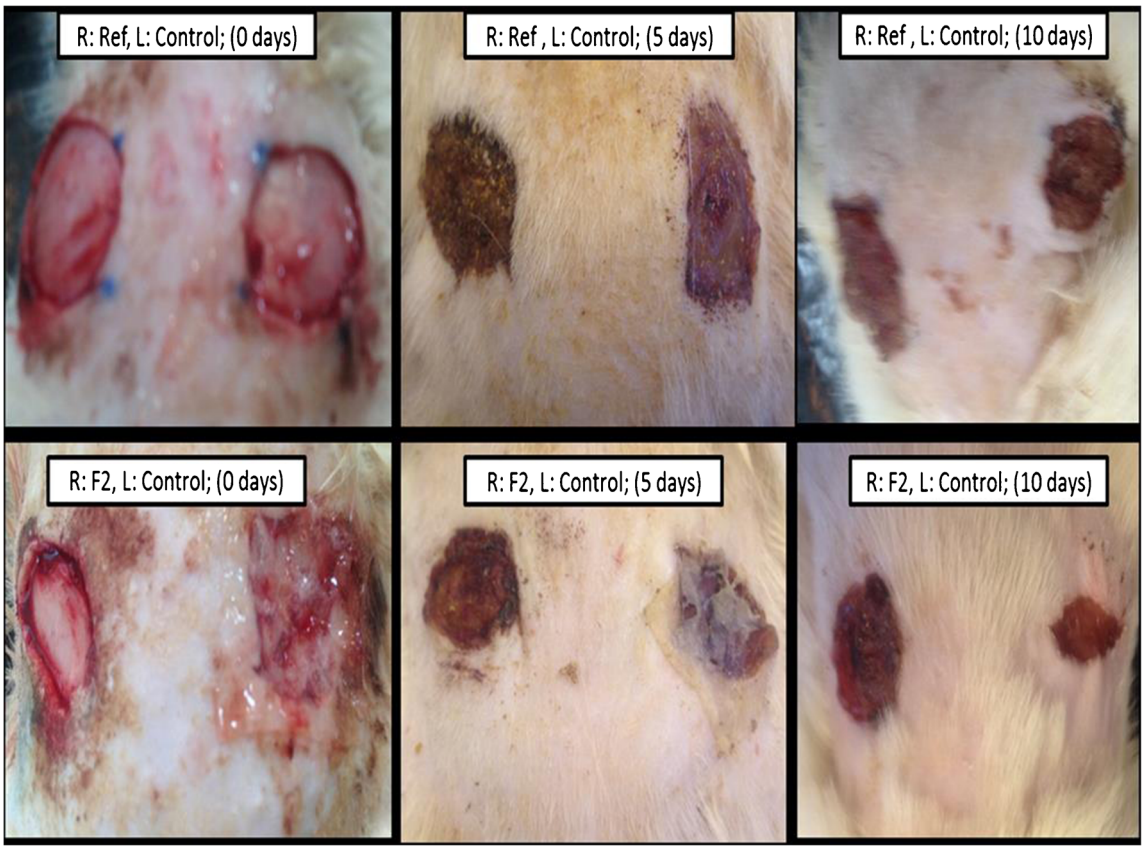

Fig. 7. Representative photographs of wound spot at 0, 5, and 10 days of treatment, for F2 and the reference

2. Log of cumulative drug remaining *versus* time (first-order kinetic model).
3. Cumulative drug released *versus* square root of time (Higuchi model).
4. Log cumulative drug released *versus* log time was plotted to study the mechanism of the drug release according to the Korsmeyer model.

The kinetics of metronidazole release from the candidate formula F3 hydrogel was determined by the multiple coefficients (r^2) for each individual product (Table III).

The n value which is used to characterize different release mechanisms in the Korsmeyer-Peppas model represents one of the following cases:

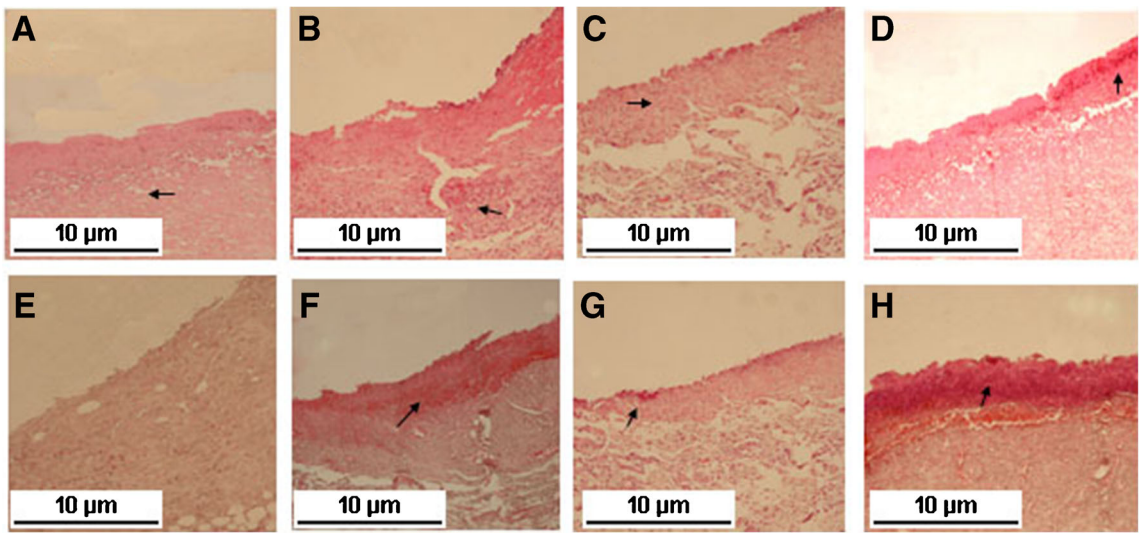

Fig. 8. Representative histopathological profiles of skin wounds of **a** control, **b** conventional product, **c** hydrogel with no drug, and **d** hydrogel with 0.1% drug-treated groups on day 5 postoperation; the inflammation is shown by *arrows*. Figures **f–h** represent the wound area 10 days postoperation and epithilization is shown by *arrows*. H&E stain ×100

1. Diffusion exponent (n) equal to 0.44 means Fickian diffusion.
2. $0.45<n<0.89$ means anomalous (non-Fickian) diffusion.
3. $n=0.89$ means case-II transport.
4. $n>0.89$ means super case-II transport.

Our results suggested that the mechanism of metronidazole release from F3 followed Fickian diffusion ($n=0.438$) which is drug concentration dependent. However, the value of n obtained for F2 was greater than 0.5, indicating the anomalous drug release from the hydrogel by both diffusion and polymer erosion mechanisms.

Thermal Analysis of Films

The purpose of the study was to investigate drug-polymer interaction. Figure 5 shows the DSC thermograms of metronidazole alone, sodium alginate polymer, physical mixtures of sodium alginate and the drug, as well as metronidazole-loaded wound dressings. A sharp endothermic peak corresponding to the melting point of crystalline metronidazole was found at 168.9°C, while sodium alginate showed a broad endothermic peak at 119.9°C due to loss of water and decomposition at 260.6°C with a strong exotherm which might be due to the decomposition of biopolymer and the formation of respective carbonate (34). The melting endotherm of metronidazole in the physical mixture and in the wound dressing formula appeared at 163.1°C.

There are slight differences observed in the melting endotherms and peak intensities which may be attributed to smaller amount of drug per sample of film analyzed compared to the weight of pure drug and physical mixtures measured.

FTIR Spectroscopy

FTIR spectroscopy was carried out in order to detect any peak shift that could be attributed to weak interactions between the drug and the polymer, such as hydrogen bonding or complexation. The FTIR spectra of the films showed spectral patterns similar to those of the pure drug and polymer, but the bands appear at shifted positions.

The polymer and drug showed different characteristic functional group FTIR bands as published in the literature (25,35) (Fig. 6). The bands of sodium alginate appeared at 3,500 cm^{-1} for the hydroxyl groups and at 1,613 and 1,405 cm^{-1} for the asymmetric –COO– stretching vibration and symmetric –COO– stretching vibration, respectively. On the other hand, metronidazole has characteristic peaks at 3,216 cm^{-1} for intramolecular hydrogen bonding of O–H stretching. The peak 1,532 cm^{-1} is for the C–NO_2 for the symmetric stretching frequency. The spectrum of the blended films has undergone a step transition resulting in a band shift to a lower wave number. The shift might be attributed to the formation of hydrogen bonding between the nitrogen atoms of metronidazole and the hydroxyl groups of alginate.

In Vivo Wound-Healing Study

Each wound was observed for a period of 5 and 10 days postoperation. All rats survived throughout the postoperative period until sacrifice. The healing process for each wound treated by dressing application progressed satisfactorily without any apparent complications. There were no evidences of necrosis. At day 5 postoperatively, little discrete inflammation was observed (Fig. 7). There was no evidence of infection or contraction of the wound, whereas the skin was hemorrhagic for some control samples and also scab was present on the wound spot. There was a significant ($p<0.05$) reduction in wound size of group 1 (F2 treatment) in comparison to group 2 (reference). At 10 days postoperatively, majority of the wounds appeared to be healed. This observation may be attributed to the ability of those dressings to protect the wound and create an environment favorable to healing (36).

Histopathology

Healing pattern of wounds was studied by the histology of control, conventional drug, hydrogel without drug, and hydrogel with drug at 5 and 10 days postoperatively (Fig. 8a–h).

At 5 days postoperatively, the skin was hemorrhagic in the control animals and few scabs spots were present on the wound in the control animals with no reduction in wound size in all groups. At day 10, the wounds almost appeared to be healed and wound size reduction was significantly greater in the order of hydrogel with drug > hydrogel without drug > conventional product > control. This may be due to the ability of alginate dressing to maintain an adequate blood flow below the level of injury and create an environment favorable to wound healing (36). This observation had been also reported by Nicholson and Armstrong (37) with the use of 10% topical metronidazole.

On the fifth day of postoperation, histology analysis showed that the test and control wounds showed different degrees of inflammation which is essential in the healing process. Gross inflammation was seen in the hydrogel-treated groups (with and without drug). Results suggested that a wet environment would enhance wound healing at day 5 postoperation. At day 10, reepithelialization of desquamated epithelial areas was extensively observed in the hydrogel with drug and conventional product compared with the hydrogel without drug and the control group, while the control wounds did not completely recovered and still showed inflammatory cell.

CONCLUSION

The metronidazole-loaded wound dressing developed using by combined Ca^{2+} cross-linking and freeze-thawing cycle method was more swellable with better capability to adsorb protein. The drug release was obtained with F3 for a longer time compared to F2 and the reference product. The hydrogel (F2) composed of 6% sodium alginate and 1% drug with Ca^{2+} cross-linking improved the wound-healing effect compared with the hydrogel without drug, and the conventional product. Hence, this metronidazole-loaded hydrogel could be a potential candidate for controlled drug release and is expected to help to achieve rapid wound healing due to the incorporation of metronidazole as an antibacterial agent.

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