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INTRODUCTION

Incidences of Oropharyngeal candidiasis (OPC) are increasing worldwide and are most common with *Candida albicans*[1]. The clinical symptoms of oropharyngeal candidiasis can be sensations, altered taste, and altered smell, which may affect the patient's quality of life[2]. A number of effective antifungal drugs could be used either topically or systemically for management of OPC. Clotrimazole (CZ) is a lipophilic antimycotic drug with a broad spectrum utilized for treatment of fungal infections[3-4] which is mainly used in the management of superficial candidiasis. Nevertheless, it has poor aqueous solubility (0.49 μg/ml)[5] which might affect its antimycotic activity. Therefore, enhancement of solubility and release rate of the CZ is necessary for a desirable and rapid antimycotic activity. Fast-dissolving drug delivery systems have played an important role since their advantages, such as enhancing drug solubility, onset of action and bioavailability[6]. Polyvinylpyrrolidone (PVP) is one of the polymers which can provide this feature as it offers the possibility of ultrafast dissolution. Complexation of poorly soluble drugs with cyclodextrin is a useful method to improve the dissolution and stability of the drugs[7]. Nanofibers are used in many fields because they exhibits outstanding characteristics such as high porosity and specific surface area and very small pore size[8]. Electrospinning is widely used to produce nanofibers because of simple and easy way to control the morphology of ultrafine fibers[9]. In this work, CZ-loaded electrospun nanofiber mats using HPβCD functionalized PVP as the filament-forming polymer were developed. The properties of the electrospun nanofiber and the drug release characteristics were investigated. Moreover, the nanofiber mats were also investigated for antifungal activity and cytotoxicity.

MATERIALS AND METHODS

Material Clotrimazole, HPβCD, PVP (MW. ~1,300,000) and 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT), Sabouraud dextrose broth. Dimethyl sulfoxide (DMSO) were purchased from various suppliers. The human gingival fibroblast (HGF) was obtained from the Faculty of Dentistry, Naresuan University, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin-streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen. All other reagents and solvents were of analytical grade and used without further purification.

Electrospinning of CZ-loaded PVP/HPβCD nanofibers 8% PVP and 70 mM HPβCD were dissolved in a solvent mixture of EtOH: H_2O: BzOH with a volume 70:20:10. Clotrimazole (5%, 10%, 15% and 20%wt to polymer) was added into the mixture and stirred for 12 h at room temperature. The viscosity and conductivity of these mixed solutions were determined. The electrospinning process was conducted at 25 °C with the fix applied voltage, the distance between the tip and the collector, and the feeding rate as 15 kV, 15 cm and 0.3 ml/h, respectively.

Characterization of CZ-loaded PVP/HPβCD nanofibers The morphology and diameter, chemical structure, thermal behavior and physical status were investigated using scanning electron microscope (SEM), Fourier Transform Infrared spectrophotometer (FT-IR), Differential scanning calorimeter (DSC) and X-ray diffractometer (XPRD), respectively.

CZ content and In vitro release The total content of CZ in the CZ-loaded nanofiber mats and *In vitro* release studies were determined in triplicate using HPLC. The *In vitro* release studies were modified from the dissolution test which is official in USP (USP 35, 2011)[10]. Briefly, 5 mg of the CZ-loaded nanofiber mats were placed in bottle containing 50 ml of 0.1 N HCl solution (pH 1.2) incubated at 37°C under shaking at 150 rpm. After a given time, an aliquot of the release medium solution was analyzed.

Antifungal activity of the nanofiber mats

Susceptibility testing A broth microdilution method in accordance with the guidelines recommended by CLSI (Clinical and Laboratory Standards Institute, 2002), using serial two-fold dilutions of CZ in Sabouraud dextrose medium, was employed to determine susceptibility of the *C. albicans* and *C.*

dubliniensis to clotrimazole. Briefly, *C. albicans* and *C. dubliniensis* were incubated (10^4 CFU/ml) in 48 well plates for 48 h at 37 °C exposed to serial 2-fold dilution in the SDB culture medium of the CZ solution (from 200 to 0.4 µg/ml).

Time-kill analysis The rate of killing of fixed inoculums of *C. albicans* and *C. dubliniensis* (usually 10^4 CFU/ml) is determined by incubating with agitation the test organism in SDB medium containing different concentration of CZ form nanofiber mats, powder and lozenges (10 mg, Candinas troche; Thailand Jan Laboratories). The aliquot of control and CZ containing medium were removed and diluted at the 5, 15, 30, 60, 120 minute time point and then determining the survivor colony count (CFU/ml). The kill curves are constructed by plotting the CFU/ml surviving at each time point in the presence and absence of the nanofiber mats, CZ powder and lozenges.

Evaluation of cytotoxicity The cytotoxicities of the CZ and CZ-loaded nanofiber mats were evaluated using an MTT cytotoxicity assay. The human gingival fibroblast cells (HGF) which obtained form explants of gingival tissue attached to non-carious, freshly extracted third molar from three patients were used. The HGF cells were plated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids and 0.1% penicillin-streptomycin and were distributed at a density of 10,000 cells/well in 96-well plates. The cells were grown under humidified atmosphere until confluency. The cells were treated with CZ at various concentrations ranging from 25 to 400 µg/ml in serum-free medium and incubated for 24 h. For the CZ-loaded nanofiber mats, cytotoxicity test performing based on a procedure adapted from the ISO10993-5 standard test method (indirect contact). The CZ (0-20%)-loaded nanofibers were sterilized by UV radiation for 1 h and then immersed in a serum-free medium in an incubator for 24 h to produce extraction media of varying concentrations. The tested extraction media at varying concentrations were replaced and the cells were re-incubated for 2 h and 24 h. After treatment, the serum-free medium containing CZ and the tested extraction solutions were removed. Finally, the cells were incubated with 100 µl of an MTT-containing medium (1 mg/ml) for 4 h. The medium was removed, the cells were rinsed with a phosphate buffer (pH 7.4), and the formazan crystals formed in the living cells were dissolved in 100 µl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm determined using a microplate reader.

Statistical analysis All experimental measurements were collected in triplicate. The values are expressed as the mean ± standard deviation (SD). The statistical significance of the differences in each experiment was examined using one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) post hoc test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

PVP/HPβCD nanofiber mats Electrospinning of PVP/HPβCD solution containing various amounts of CZ (0, 5, 10, 15 and 20%wt CZ to polymer) were carried out from EtOH:H_2O:BzOH (70:20:10). The average fiber diameter of nanofiber electrospun from solution containing CZ content as 0, 5, 10, 15 and 20% wt to polymer was 665, 663, 667, 657 and 645 nm, respectively. Thus, high amount of CZ can be incorporated in nanofiber mats without any effect on the fiber diameter.

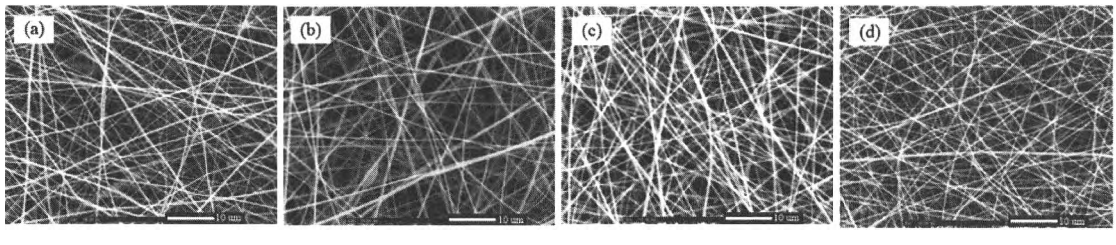

Figure 1 The SEM image (1,000x) of the PVP/HPβCD nanofiber mats with a different CZ loading amount; a) 5, b) 10, c) 15 and d) 20%wt CZ to polymer

The FT-IR spectrum show that pure CZ powder exhibits dominant absorption peaks at 1,586.7, 1,490.7, and 1,304.9 cm^{-1} corresponded to the benzene ring stretching. The band at 904.7, 823.68, and 744.59 cm^{-1} are assigned to the C-H stretching. The band 1,081.4 cm^{-1} and 1,210.1 cm^{-1} correspond to the chlorobenzene and C-N stretching, respectively. The peak that was observed in the CZ powder spectrum was also observed in spectra of the 5-20% CZ-loaded nanofiber mats. Thus, CZ was well incorporated into the nanofiber mats.

The thermogram of CZ powder exhibits an endothermic sharp peak at 145.13 °C due to melting temperature of CZ. The endothermic curves of the pure nanofiber mat (0% CZ) were also observed at

167.38 °C. The melting point slightly increased to 173.59, 177.12, 178.12 and 186.49 °C when the concentration of CZ increased to 5, 10, 15 and 20%, respectively. The absence of detectable crystalline domain, even at high content of CZ, indicates that CZ was incorporated in PVP/HPβCD nanofiber mats in the amorphous state. These results are in accordance with XRPD result which showed that no peak is found in the diffractograms of nanofiber mats containing various amounts of CZ.

Drug content and release studies The entrapment efficiency of CZ in the nanofiber mats was 92.60-96.85%, which shows the excellent incorporation of CZ in the nanofiber mats. An increasing of the initial amount of CZ resulted in an overall increase in the amount of CZ entrapped in the nanofiber mats. The loading capacity of CZ in the nanofiber mats was increased from 0.046 to 0.161 mg/mg of nanofiber when increasing the initial amount of CZ from 5% to 20% wt to polymer.

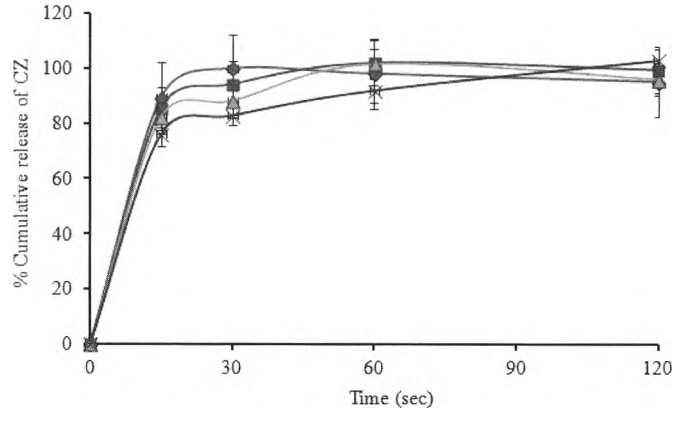

Figure 2 Release profiles of CZ from the CZ-loaded PVP/HPβCD nanofiber mats with different amounts of CZ: (◆) 5%, (■) 10 %, (▲) 15 % and (●) 20 % to polymer. The data are expressed as mean ± standard deviation from three independent experiments. * Statistically significant ($P<0.05$).

The release characteristics of CZ from the CZ-loaded PVP/HPβCD nanofiber mats with different percentages of CZ are shown in Figure 2. It was observed that the CZ was rapidly released from the CZ-loaded electrospun nanofiber mats. After 30, 60 ,60 and 120 second, all of CZ contained in the 5, 10, 15 and 20% CZ-loaded nanofiber mats were released into dissolution medium respectively. The fast release characteristic of CZ from the nanofibers mat is due to the extremely high specific surface area and porosity, which promoting a remarkably fast drug release, and the ability of PVP nanofiber to provide a fast-dissolving hydrophilic environment. Moreover, the electrospinning process changes the crystalline stage of CZ into amorphous state which facilitates dissolution of the drug in dissolution medium.

Antifungal study The MIC and MFC values of CZ are obtained from susceptibility testing using a broth dilution assay. It is evident that CZ is strongly active against *C. albicans* and *C. dubliniensis* with the MIC value of 12.5 and 6.25 µg/ml, respectively; besides, the MFC were 25 and 12.5 µg/ml, respectively.

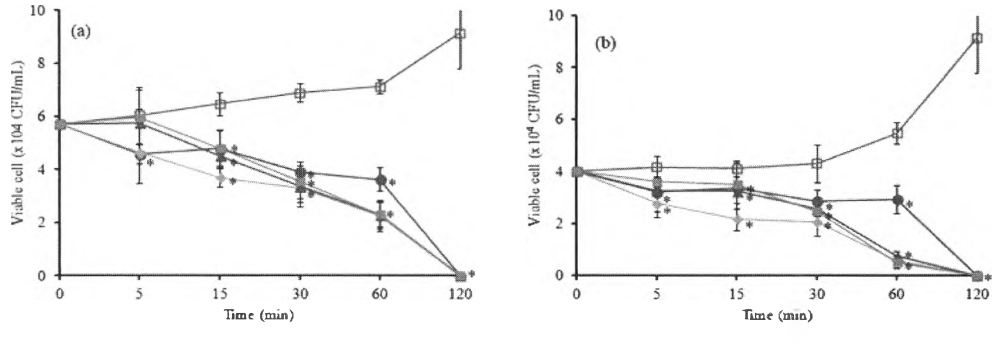

Figure 3 Time kill plot of a) *C. albicans* and b) *C. dubliniensis*. (CFU/ml) versus the treatment time: (□) the control, (●) 5 mg of 20% CZ-loaded PVP/HPβCD nanofiber mats, (◆) 20% CZ-loaded PVP/HPβCD nanofiber mats equivalent to CZ 1 mg/ml, (▲) CZ powder equivalent to CZ 1 mg/ml and (■) CZ lozenges equivalent to CZ 1 mg/ml. The data are expressed as mean ± standard deviation from three independent experiments. * Statistically significant ($P<0.05$).

The antifungal activity of CZ loaded nanofiber mats against *C. albicans* and *C. dubliniensis* was tested by counting the viable bacterial cells that rested in a *Candida* suspension after the contact of the nanofiber mats with the suspension. About 10^4 CFU/ml of the strain was exposed to the nanofiber mat with different CZ loading; furthermore, the CZ-loaded nanofiber mat which gives a final concentration of CZ equivalent to 1mg/ml in *Candida* suspension was also tested for time kill assay in comparison to the CZ powder and the lozenges at the same concentration of CZ. The CZ-loaded nanofibers have ability to reduce the *Candida* and killed within 120 min of contact. In contrast, the pure PVP/HPβCD nanofiber did not reduce the *Candida* growth. In comparison to the CZ powder and CZ lozenges, the CZ-loaded nanofiber which gives the same final CZ concentration can significantly reduce exhibit faster activity to kill the *Candida* than CZ powder and the lozenges (Figure 3) due to a very fast disintegration of the nanofiber and the amorphous state of CZ in nanofiber which can fast and easy dissolve than the crystalline of the CZ powder. The CZ lozenges must take time to disintegrate and follow by dissolving; thus, it also shows slow antifungal activity. The fast antifungal activity of the CZ-loaded PVP/HPβCD nanofiber mats render them promising for oral candidiasis application.

Cytotoxicity evaluation The cytotoxicity effect of various concentrations of CZ and the extraction medium from 0-20% loaded nanofiber mats in HGF cells were determine as % viability (Figure 4a). A concentration-dependent cytotoxicity of the CZ was observed for a 24-h incubation period. The IC_{50} values, indicating 50% cell deactivation, of CZ were greater than 25 μg/ml. For the CZ-loaded nanofiber mats, There was a significant decrease in cell viability when the HGF cells were incubated with the higher loading amount of CZ in the nanofiber mat (0-20%) when compared to control ($p<0.05$).

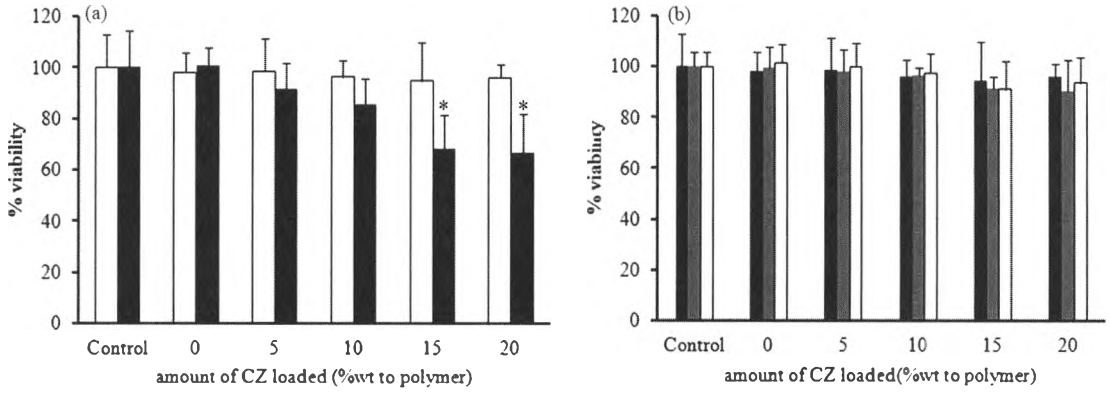

Figure 4 The percentage cell viability in HGF cells of the CZ loaded PVP/HPβCD nanofiber mats containing 0, 5, 10, 15 and 20%wt CZ to polymer: a) incubated at (□) 2 h and (■) 24 h and b) incubated at 2 h in HGF cells derived from 3 patients: (■) patient #1, (▒) patient #2 and ( ) patient # 3. Each value represents the mean ± standard deviation of five wells. * Statistically significant (P<0.05).

The cytotoxicity was observed after 24-h incubating with the extraction media of the nanofiber mats containing high content of CZ (15–20 % wt. to polymer) (Figure 4a). However, the cytotoxicity of CZ was reduced after incorporated in nanofiber mat. For the 2-h incubates the extraction media of the nanofiber mats with different concentration of CZ, the cell viability remained similar to that of the control cells in all concentrations. The result showed no significant difference in cytotoxicity of the CZ-loaded nanofiber mats in HGF cells derived from three different patients as shown in Figure 4b.

CONCLUSION

The CZ-loaded PVP/HPβCD nanofiber mats were successfully prepare by electrospining using EtOH: H_2O: BzOH with a ratio of 70:20:10 as a solvent system. An increasing of CZ content did not affect on the diameter of the nanofiber mats. The rapidly released CZ from the CZ-loaded electrospun nanofiber mats were achieved. The nanofiber mats exhibit faster antifungal activity than CZ powder and CZ lozenges. Moreover, the cytotoxicity of CZ was reduced after incorporated in nanofiber mat. Hence, these nanofiber mats may be promising for oral candidiasis application.

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