Annals of Microbiology, **57 (1)** 115-119 (2007)

In vitro inhibitory activity of EDTA against planktonic and adherent cells of *Candida* sp.

Beata CHUDZIK[1]*, Anna MALM[1], Barbara RAJTAR[2], Malgorzata POLZ-DACEWICZ[2]

[1]Department of Pharmaceutical Microbiology, [2]Department of Virology, Medical University of Lublin, 1 Chodzki Street, 20-093 Lublin, Poland

Received 13 September 2006 / Accepted 10 January 2007

Abstract - *Candida* sp. can cause infections of indwelling medical devices associated with biofilm formation, which are difficult to treat due to insensitivity of adherent microorganisms to host defence mechanisms and standard antimicrobial therapy. The aim of this paper was to determine the effect of EDTA (disodium salt) on the adhesion of *Candida* sp. to some catheters and also on biofilm formation by the yeasts and its eradication in relation to cytotoxicity of this chelating agent to the cell cultures. The adhesion process and biofilm formation, and also EDTA cytotoxicity to green monkey kidney (GMK) cell culture were determined using MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] reduction assay. EDTA inhibited the growth of free-floating forms of *Candida* sp. strains with minimal inhibitory concentration (MIC) from 0.06 to 0.25 mM; the minimal fungicidal concentration (MFC) values ranged from 64 to 128 mM. The prevention of *Candida* sp. adhesion on the catheters used or eradication of the adherent cells was achieved at 0.5 to 4.0 mM EDTA. Also biofilm formation was prevented by 0.5 to 4.0 mM EDTA. Much higher concentration of EDTA (32 to 128 mM) was needed to eradicate the mature biofilm. EDTA at concentration up to 1 mM was not toxic for GMK cells. At higher concentration, toxicity of EDTA to GMK cells was correlated with the concentration of this agent and the time of exposure. Summing up, EDTA may be regarded as a useful agent rather in prophylaxis of candidal infections of medical devices.

Key words: *Candida* sp., biofilm, EDTA, biomaterials.

INTRODUCTION

The yeast-like fungi belonging to the genus of *Candida* can cause a variety of superficial and deep-seated mycoses. Recently, these microorganisms have been involved in nosocomial infections associated with indwelling medical devices, complicated by catheter-related blood-stream infections (Raad *et al.,* 2003; Ramage *et al.,* 2005). The devices become colonised by the yeast-like fungi that form a biofilm – an universal, complex, interdependent community of surface-linked microbial cells embedded in the matrix of extracellular polymeric substances (Hawser and Douglas, 1994; Jabra-Rizk *et al.,* 2004). Biofilm-associated infections are difficult to treat due to the inherent resistance of adherent microorganisms both to host defence mechanisms and standard antimicrobial therapy (Baillie and Douglas*,* 1999). Although, *Candida albicans* is predominant etiologic agent of candidiasis, other species that tend to be less susceptible to the commonly used antifungal drugs such as *Candida krusei, Candida glabrata* or *Candida famata* have emerged as opportunistic pathogens (Chandra *et al.,* 2001; Jabra-Rizk *et al.,* 2004).

EDTA is a known metal chelator, showing *in vitro* inhibitory activity against *Candida* sp. (Gil *et al*., 1994). Several authors have studied the efficacy of EDTA (disodium or tetrasodium salt) alone or in combination with other agents (*e.g.* minocycline) against clinically relevant microorganisms, most commonly isolated from hospital required medical device-related infections, including *Candida* sp. (Kite *et al.,* 2004; Percival *et al.,* 2005; Raad *et al.,* 1997, 2003, 2006). The aim of this paper was to determine the effect of disodium EDTA on adhesion of cells of various species of *Candida* sp. to surfaces of some catheters and also on biofilm formation by the yeasts and its eradication in relation to cytotoxicity of this chelating agent to the cell line cultures.

MATERIALS AND METHODS

Microorganisms and culture conditions. A total of 7 strains of *Candida* sp., possessing hydrophilic or hydrophobic cell surface, were used in this study. The hydrophobicity of cell surface was assessed using Salt Aggregation Test (SAT) according to Lindahl *et al.* (1981). The collection included the following isolates: *C. albicans* (1 hydrophilic and 2 hydrophobic isolates), *C. famata* (1 hydrophilic and 1 hydrophobic isolates), *C. glabrata* (1 hydrophobic isolate), *C. krusei* (1 hydrophilic isolate). The isolates were obtained from nasopharynx of patients with lung cancer undergoing pulmonary resection, stored at –20 °C in 50% glycerol and cultured on Sabouraud dextrose agar at 30 ºC for 48 h;

* * Corresponding author. E-mail: BeChudz@poczta.fm

before each experiment, the isolates were subcultured on Sabouraud glucose broth (further called Sabouraud medium) at 30 ºC for 48 h.

EDTA. Standard powder of EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate, approx. 99% titration) was examined (Sigma).

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) for EDTA. Determination of MIC for EDTA was performed by a broth microdilution method in accordance with the guidelines recommended by CLSI (Clinical and Laboratory Standards Institute, 2002), using serial two-fold dilutions of EDTA in Sabouraud medium. Final concentrations of EDTA ranged from 0.03 to 128 mM. Stock inoculum suspensions of yeasts were prepared in Sabouraud medium and adjusted to optical density corresponding to 0.5 Mc Farland standard, *i.e.* 1.5 x 10^8 CFU (Colony Forming Units)/ml. After incubation at 35 °C for 48 h, the MICs were assessed visually as the lowest EDTA concentration showing complete growth inhibition. In order to determine the MFC for EDTA, 10 µl from each tube that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto Sabouraud dextrose agar plates. After the incubation at 35 °C for 48 h, the MFCs were assessed visually as the lowest EDTA concentration from ≤ 1 colony was visible on the agar plate (Pujol *et al*., 2000). All experiments were done in triplicates. The representative data are presented.

Biomaterials. All assays were carried out on two types of catheters that differed in unevenness of surface from each other, silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter.

The effect of EDTA on adhesion of *Candida* sp. and biofilm formation on the biomaterials. The adhesion process and biofilm formation were determined by using MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay (Levitz *et al*., 1985). The used catheters were cut aseptically into ca 0.5 cm^2 fragments and placed into Petri dishes. The standardised yeast suspensions (optical density of 0.5 Mc Farland standard) were prepared. In each experiment, various concentrations of EDTA (0.03-128 mM) were used.

(i) In order to assay the effect of EDTA on adhesion process, the yeast suspensions in sterile PBS (phosphate-buffered saline) containing EDTA were incubated with biomaterials at 35 °C for 1 h. Nonadherent cells were removed by careful rinsing catheter discs with sterile phosphate-buffered saline (PBS) and then resuspended in Sabouraud medium, followed by overnight incubation at 35 °C.

(ii) In order to assay the effect of EDTA on eradication of adherent cells, the yeast suspensions in sterile PBS were incubated with biomaterials at 35 °C for 1 h. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in Sabouraud medium containing EDTA, followed by overnight incubation at 35 °C.

(iii) In order to assay the effect of EDTA on biofilm formation, the yeast suspensions in Sabouraud medium containing EDTA were incubated with biomaterials at 35 °C for 24 h. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in fresh Sabouraud medium. Medium changing and catheters washing procedures after overnight incubation at 35°C were repeated three times (total incubation period lasted 72 h).

(iv) In order to assay the effect of EDTA on biofilm eradication, the mature 72 h-biofilms were incubated in the presence of EDTA for 24 h. In all assays a drop of 1% MTT solution was added to each dish. After incubation at 35 °C for 24 h, in the presence of *Candida* sp. viable cells, MTT was reduced to the violet tetrazolium formazan product, accompanied by violet colour of the medium. In each experiment, the control free-EDTA assays were carried out. All experiments were done in triplicates. The representative data are presented.

Cytotoxicity assay. EDTA cytotoxicity to green monkey kidney (GMK) cell was determined by MTT toxicity assay (Takenouchi and Munekata, 1998). Monolayers of GMK cells were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 100 µg/ml of streptomycin and 100 U/ml of penicillin. After overnight incubation at 37 ºC, the medium above the cell culture was removed. To the 2 ml samples of MEM without calf serum EDTA was added to obtain final concentrations ranging from 0.12 to 64 mM. Control assays containing only cells of GMK in MEM were also carried out. Cell cultures were incubated at 37 ºC for 24 or 72 h with changing medium every day. After incubation, medium above the cell culture was removed and 1 ml of MEM without calf serum and 100 µl of 5 mg/ml MTT solution in PBS were added to the samples and the incubation was continued for another 4 h at 37 ºC before addition of 1 ml of aqueous solution containing 50% dimethylformamide and 20% solution of SDS to solubilise the insoluble formazan precipitates produced by MTT reduction. The absorbance of converted dye was measured at two wavelengths: 540 nm (A_{540}) and 620 nm (A_{620}), using ELISA plate reader (Organon Technika Microwell System Reader 530). All experiments were done in triplicates. The mean values (± SD) were presented.

RESULTS

As shown by scanning electron micrographs (data not presented), the structures of both catheters used in this study were different in the degree of surface roughness. However, we have found biofilm formation by all *Candida* sp. strains, both hydrophilic and hydrophobic isolates, on two catheters. This was monitored by formation of violet tetrazolium formazan product on inside and outside surfaces of catheters and violet coloured medium after addition of MTT solution.

According to Table 1, EDTA inhibited growth of all *Candida* sp. strains (free-floating forms) with MIC ranging from 0.06 to 0.25 mM. The MFC values for EDTA were much higher, from 64 to 128 mM, resulting in the high MFC/MIC values.

As shown Table 2, adhesion process of *Candida* sp. on both catheters was prevented from 0.5 to 4 mM EDTA. The adherent cells of *Candida* sp. were eradicated from both catheters at similar EDTA concentrations applied for 24 h. Prevention of adhesion process and eradication of adherent cells were monitored by lack of formation of violet tetrazolium formazan product after addition of MTT.

Data presented in Table 3 showed that biofilm formation by *Candida* sp. on both catheters was prevented between

TABLE 1 - *In vitro* activity of EDTA against planktonic cells of *Candida* sp.

Strain	MIC (mM)	MFC (mM)	MFC/MIC
C. albicans hydrophilic	0.062	64	1032
C. albicans hydrophobic	0.25	128 (70)*	512 (280)
C. albicans hydrophobic	0.25	128	512
C. famata hydrophobic	0.125	128	1024
C. famata hydrophilic	0.125	128 (85)*	1024 (680)
C.glabrata hydrophobic	0.125	64	512
C. krusei hydrophilic	0.25	64	256

* In parentheses detailed MFC values determined by titration at EDTA concentrations between 64 and 128 mM were presented.

0.5 and 4.0 mM of EDTA. In contrast, much higher concentrations of EDTA (32 to 128 mM) were needed to eradicate the mature biofilm of *Candida* sp. during 24-h treatment. Prevention of biofilm formation and eradication of the mature biofilm was also monitored by the lack of formation of violet tetrazolium formazan product after addition of MTT.

Table 4 shows data concerning EDTA cytotoxicity at concentration affecting adhesion of *Candida* sp. or biofilm formation and eradicating adherent cells or the mature biofilm. Using MTT cytotoxicity assay, it was found that EDTA at low concentration (up to 1 mM) was not toxic for GMK cells after 24 or 72 h treatment, as evidenced by the absorbance of the converted dye compared to that in control assay. Microscopy did not reveal any changes in the monolayer. At higher EDTA concentration, the rate of toxic effect on GMK cells monitored by decreased absorbance of the converted dye, accompanied by the destroyment of the monolayer, was proportional with concentration of the chelating agent and the time of cell treatment.

DISCUSSION

Candida sp. infections can be regarded as an important medical problem, especially in the immunocompromised patients, *e.g.* in lung cancer patients (Chandra *et al*., 2001). Besides, according to our unpublished data, *Candida* sp. isolates (*e.g. C. krusei*) have shown to possess a potential ability to colonise pleural drains in patients undergoing pulmonary resection. Although fungal infections of medical devices are less common than bacterial infections, they are most difficult to treat (Chandra *et al*., 2001).

Using MTT method, we have found out that all assayed strains of *Candida* sp. were able to form biofilm on both used catheters, irrespective of the kind of polymer (silicone, PCV) and hydrophobic or hydrophilic cell surface of the yeasts. It is known (Strevett and Chen, 2003) that several non-specific or specific interactions between microorganisms and an array of surfaces, including biomaterials, are involved in microbial cell attachment and biofilm development. One of the most important factors appears to be microbial cell hydrophobicity (Liu *et al*., 2004). However, our data suggest that there is no correlation between cell surface hydrophobicity of the assayed *Candida* sp. strains and their adherent properties to the used catheters. The obtained data confirm that various species of *Candida* may be regarded as potential etiologic agents in infections associated with indwelling medical devices.

EDTA is known to possess anticoagulant properties. Besides, this chelating agent is recommended for some therapeutic purposes, *e.g.* hypercalcaemia at lead poisoning (Raad *et al.,* 1997) or endodontic therapy in patients with oral candidiasis (Sen *et al*., 2000). In addition, EDTA, possessing anticandidal activity *in vitro* (Gil *et al.,* 1994), may be regarded as a potential agent in prophylaxis and treatment of infections of medical devices.

Data presented in this paper indicate that growth of planktonic (free-floating) *Candid*a sp. was inhibited by EDTA at concentration ranging from 0.06 to 0.25 mM. It should be noted that MIC values for EDTA for the assayed *Candida* sp. clinical isolates were comparable to those described by other authors for clinical isolates of the yeasts (Raad *et al.,* 1997). In contrast, the obtained MFC values were much

TABLE 2 - *In vitro* effect of EDTA on adhesion process of *Candida* sp. to biomaterials determined by MTT assay

Strain	Minimal concentration preventing adhesion process (mM)		Minimal concentration eradicating adhesion process (mM)	
	Thorax catheter	Foley catheter	Thorax catheter	Foley catheter
C. albicans hydrophilic	2	2	2	2
C. albicans hydrophobic	0.5	2	0.5	1
C. albicans hydrophobic	4	4	4	4
C. famata hydrophobic	2	4	2	4
C. famata hydrophilic	0.5	0.5	1	1
C.glabrata hydrophobic	0.5	2	2	2
C. krusei hydrophilic	1	2	1	2

TABLE 3 - *In vitro* effect of EDTA on biofilm-embedded cells of *Candida* sp. determined by MTT assay

Strain	Minimal concentration preventing biofilm formation (mM)		Minimal concentration eradicating biofilm (mM)	
	Thorax catheter	Foley catheter	Thorax catheter	Foley catheter
C. albicans hydrophilic	2	2	64	64
C. albicans hydrophobic	0.5	0.5	64	64
C. albicans hydrophobic	2	4	128	128
C. famata hydrophobic	2	4	64	64
C. famata hydrophilic	2	2	128 (80)*	32
C.glabrata hydrophobic	2	2	32	32
C. krusei hydrophilic	2	2	64	64

* In parenthesis EDTA concentration determined by titration between 64 and 128 mM were presented.

TABLE 4 - Effect of EDTA on viability of GMK cells determined by MTT assay

EDTA conc. (nM)	Viability			
	After 24 h		After 72 h	
	540 nm	620 nm	540 nm	620 nm
None	1.502 ± 0.067*	1.005 ± 0.045	1.593 ± 0.168	1.032 ± 0.095
0.25	1.581 ± 0.196	1.041 ± 0.124	1.614 ± 0.111	1.046 ± 0.067
0.5	1.421 ± 0.020	0.947 ± 0.015	1.481 ± 0.179	0.918 ± 0.089
1	1.458 ± 0.032	0.962 ± 0.006	1.530 ± 0.044	1.005 ± 0.029
2	0.924 ± 0.216	0.581 ± 0.129	0.649 ± 0.159	0.394 ± 0.096
4	0.496 ± 0.058	0.300 ± 0.042	0.242 ± 0.011	0.117 ± 0.005
32	0.395 ± 0.013	0.264 ± 0.004	0.103 ± 0.004	0.077 ± 0.022
64	0.259 ± 0.061	0.185 ± 0.041	0.049 ± 0.025	0.006 ± 0.002
128	0.231 ± 0.073	0.170 ± 0.037	0.045 ± 0.005	0.005 ± 0.002

* Values are mean ± SD.

higher in comparison to those presented in the literature (Raad *et al.,* 1997). This suggests differential, strain-dependent fungicidal effect of EDTA against *Candida* sp.

The growth of the assayed *Candida* sp. in presence of the fragments of catheters was inhibited at concentration somewhat higher than MIC values, ranging from 0.5 to 4.0 mM. This is in agreement with the general view that the adherent forms of microorganisms become resistant to agents that are active against the planktonic forms of the same organism (Hawser, 1996; Baillie *et al.,* 1999; Chandra *et al.,* 2001; Raad *et al.,* 2003; Jabra-Rizk *et al*., 2004). Our data indicate that eradication of the preformed, mature biofilm of *Candida* sp. required high concentrations of EDTA (32 to 128 mM) comparable to those showing fungicidal activity for planktonic cells of the yeasts. Also other authors (Percival *et al.*, 2005; Raad *et al.,* 1997, 2003) found that di- or tetra-sodium EDTA alone might be effective in eradication of catheter-associated biofilms of *C. albicans* at concentration 30 mg/ml (about 81 mM) or 40 mg/ml (about 108 mM). However, according to our data, the high concentration of EDTA showed toxic effect on viability of cell cultures. On the other hand, in special clinical cases, *e.g.* treatment of hypercalcaemia at lead poisoning EDTA was used at much higher concentration – about 8.1 M (Raad *et al.,* 1997). It should be noted that eradicating EDTA effect against microorganisms (*e.g. C. parapsilosis*) embedded in biofilm could be potentiated by combination of this agent with minocycline or with minocycline and 25% ethanol due to their synergistic activity (Raad *et al*., 1997, 2006).

It is possible that inhibitory activity of EDTA against adherent cells of *Candida* sp. is due to its inhibitory effect on the yeast-to-mycelium transition (Gil *et al.*, 1994; Percival *et al.,* 2005). Moreover, the well-known metal-chelating activity of EDTA may be primarily involved in inhibition of *Candida* sp. attachment to biomaterials. Our data suggest that there is no correlation between cell surface hydropho-

bicity or hydrophilicity of the assayed *Candida* sp. strains, even among strains belonging to the same species, *e.g*. two hydrophobic isolates of *C. albicans*. Also, the sensitivity to EDTA of the yeasts in the form of planktonic, adherent or embedded in biofilm cells is not correlated with cell surface properties. However, these problems require further studies.

Several studies were undertaken to manage microbial biofilm formation on biomaterials, including the incorporation of antimicrobial agents into biomaterials, changing surface chemistry of biomaterial or lock treatment (Raad *et al.*, 1997, 2003, 2006; Percival *et al.,* 2005). Our data and those from literature indicate that EDTA, at relatively low concentration, may be useful in preventing colonisation by both *C. albicans* and non-*albicans Candida* sp. of various type catheters, and hence in prophylaxis of candidal infections associated with indwelling medical devices. Besides, the MTT tetrazolium assay is a simple and useful method in the estimation of adhesion and biofilm formation by *Candida* sp. and in screening the assessment of effects of various agents on these processes.

Data presented in this paper indicate that EDTA may be regarded as a useful agent rather in prophylaxis of candidal infections of medical devices but not in the eradication of the preformed *Candida* sp. biofilm due to its cytotoxicity to cell cultures at higher concentration.

Acknowlegments

This work was supported by a grant from European Social Found (Agreement No. Z/2.06/II/2.6/09/04/U/06/04).

REFERENCES

Baillie G.S., Douglas L.J. (1999). *Candida* biofilms and their susceptibility to antifungal agents. Method Enzymol., 310: 644-656.

Chandra J., Kuhn D.M., Mukherjee P.K., McCormick T., Ghannoum M.A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance**.** J. Bacteriol., 183: 5385-5394.

CLSI - Clinical and Laboratory Standards Institute (2002). NCCLS document M27-A2. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard-Second Edition, Wayne, Pennsylvania, USA.

Gil M.L., Casanova M., Martinez J.P. (1994). Changes in the cell wall glycoprotein composition of *Candida albicans* associated to the inhibition of germ tube formation by EDTA. Arch. Microbiol., 161: 489-494.

Hawser S.P., Douglas L.J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. Infect. Immun., 62: 915-921.

Hawser S. (1996). Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised *C. albicans* cells. J. Med. Vet. Mycol., 34: 149-152.

Jabra-Rizk M.A., Falkler W.A., Meiller T.F. (2004). Fungal biofilms and drug resistance. Emerg. Infect. Dis., 10: 14-19.

Kite P., Eastwood K., Sugden S., Percival S.L. (2004). Use of *in vivo*-generated biofilms from hemodialysis catheters to test the efficacy of a novel antimicrobial catheter lock for biofilm eradication *in vitro*. J. Clin. Microbiol., 42: 3073-3076.

Levitz S.M., Diamond R.D. (1985). A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J. Infect. Dis., 152: 938-944.

Lindahl M., Faris A., Wadstrom T., Hjerten S. (1981). A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophys. Acta, 677: 471-476.

Liu Y., Yang S.F., Li Y., Xu H., Qin L., Tay J.H. (2004). The influence of cell and substratum surface hydrophobicities on microbial attachment. J. Biotechnol., 110: 251-256.

Percival S.L., Kite P., Eastwood K., Murga R. (2005). Tetrasodium EDTA as a novel central venous catheter lock solution against biofilm. Infect. Control Hosp. Epidemiol., 26: 515-519.

Pujol I., Aguilar C., Fernándes-Ballart J., Guarro J. (2000). Comparison of the minimum fungicidal concentration of amphotericin B determined in filamentous fungi by macrodilution and microdilution methods. Med. Mycol., 38: 23-26.

Raad I., Buzaid A., Rhyne J., Hachem R., Darouiche R., Safar H., Albitar M., Sherertz R.J. (1997). Minocycline and ethylenediaminetetraacetate for the prevention of recurrent vascular catheter infections. Clin. Infect. Dis., 25: 149-151.

Raad I., Chatzinikolaou I., Chaiban G., Hanna H., Hachem R., Dvorak T., Cook G., Costerton W. (2003). *In vitro* and *ex vivo* activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. Antimicrob. Agents Chemother., 47: 3580-3585.

Raad I., Hanna H., Dvorak T., Chaiban G., Hachem R. (2006). Optimal antimicrobial catheter lock solution, using different combination of miocycline, EDTA and 25% ethanol: rapid eradication of organisms embedded in biofilm. Antimicrob. Agents Chemother. Published online ahead of print on 30 October 2006.

Ramage G., Saville S.P., Thomas D.P., López-Ribot J.L. (2005). *Candida* biofilms: an update. Eukaryot. Cell, 4: 633-638.

Sen B.H., Akdeniz B.G., Denizici A.A. (2000). The effect of ethylenediamine-tetraacetic acid on *Candida albicans*. Oral Surg. Oral Med. Oral Pathol. Radiol. Endod., 90: 651-655.

Strevett K.A., Chen G. (2003). Microbial surface termodynamics and applications. Res. Microbiol., 154: 329-335.

Takenouchi T., Munekata E. (1998). Amyloid beta-peptide-induced inhibition of MTT reduction in PC12h and C1300 neuroblastoma cells: effect of nitroprusside. Peptides, 19: 365-372.