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Simple tests demonstrating the antimycotic effect of chitosan

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Summary

We show that under the conditions of a fungal growth test, 66% deacetylated krill chitosan did not affect pathogenic fungi which cause human mycosis. However, as demonstrated by our fungal cell adherence test, chitosan not only inhibited the adhesion of *Candida albicans* cells to human vaginal epithelial cells or to HeLa cells, but also acted non-specifically on fungal and epithelial cells. This explained our earlier observations of the enhancement of therapeutic efficacy of anti-fungal formulations, to which chitosan had been added. We recommend the joint use of fungal growth and fungal cell adherence tests as a generally useful, economical and readily available method for assessing the activity of anti-fungal preparations.

Introduction

This paper describes our investigation of drug formulations in which the aim was to increase their efficacy using the procedure reported by Machida and Nagai (1989). The method involved the addition of chitosan as an excipient to gel-forming powders, gels, and buccal and vaginal tablets (Knapczyk and Krówczyński, 1989). While a number of suggestions have been put forward regarding the application of chitosan, a deacylated product of chitin and its derivatives obtained from crab or krill (Sapelli et al., 1986; Muzzarelli, 1989), the first published study concerning the use of its formulations with anti-fungal drugs in human therapy for treatment of stomatitis prothetica mycotica and vaginitis mycotica was presented by the authors of the current paper (Knapczyk et al., 1989c).

Initial pre-clinical trials indicated that all of the forms used by patients were highly efficient in therapy. The criterion employed to assess the degree of success of the therapy was taken to be the observation of a negative result in mycological assays, corresponding to the disappearance or reduction of clinical symptoms and signs of mycosis. The influence of chitosan was estimated by comparing the results obtained via therapy involving the application of one tablet containing 400 mg clotrimazole and 400 mg deacylated chitosan with those determined following the use of 500 mg of a drug present in a commercial vaginal

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tablet (Liber et al., 1990). The possibility of achieving a 20% reduction in the therapeutic dose for treating mycosis of mucous membranes therefore signifies that considerable progress is being made. The previous data referred to above together with the results reported in the current article lead to the questions as to the particular role being played by chitosan in a given type of therapy and methods which may be used to clarify its action.

In view of the available literature data, the conclusion was drawn that experiments performed on animal models would not provide an answer to the problem described above, since the experimental models of fungal dermatophytosis differ between humans and other mammals. Fungal mycelia are known to grow more rapidly in the stratum corneum of the latter species than in human skin. On the other hand, in the animal models, spontaneous healing occurs without the need for an effective treatment (Yamaguchi and Uchida, 1984; Ryley, 1986). This became apparent following an attempt to interpret results observed during the therapy of surface infections in guinea pigs, some of which were left untreated whilst others were treated by the application of chitosan gels alone or in combination with clotrimazole (unpublished data).

Furthermore, chitosan, in various biochemical forms, has been found to be present in a number of pathogenic fungi, the amount being dependent upon both the degree and the duration of the infection (Uchida and Yamaguchi, 1984). In spite of the fact that chitosan is not a medicine but simply a substance that exhibits dose-dependent biological activity (Knapczyk et al., 1989a; Marcinkiewicz et al., 1991), it has been shown to be active in the treatment of tissues (Muzzarelli et al., 1988).

Determination of the particular role played by chitosan in a given medical treatment cannot be established with any certainty unless use is made of animals which have been either presented with an immunological challenge (e.g., administered with cyclophosphamide or hydrocortisone) or subjected to a treatment inducing a disease or deficiency (such as streptozotocin-induced diabetes). Such treatment thus entails considerable complications and expense in testing. Finally, mycotic diseases in humans usually stem from superinfections as a result of causes having considerable complexity in their origins (e.g., cancer, immunosuppressive or antibiotic therapy, and AIDS). Although animal models suitable for experimentation in this area are unknown, those that are available in the case of humans are both risky and costly.

The procedure employed in the current report therefore involved the combined examination of the effects of chitosan on pathogenic fungi which generate human mycotic diseases and the well-known in vitro fungal cell adherence assay method (Lehrer et al., 1983, 1986; Calderone et al., 1984; Segal et al., 1984), as modified and validated by researchers at the present laboratory (Macura, 1988, 1990; Macura and Bańkowska, 1989; Mancura and Tondyra, 1989).

Materials and Methods

Chitosan derived from krill chitin was obtained from the Sea Fisheries Institute (Gdynia, Poland). Chitosan [degree of deacetylation about 66%, MW $\simeq 10^6$, medium-viscosity type, and other properties corresponding to the known requirements (Knapczyk et al., 1989b)] was used as a powder, the grain size of less than 0.08 mm being selected using an appropriate sieve. 1% chitosan solutions of pH 4.0 and pH 5.7 were prepared by dissolving samples in dilute acetic or lactic acid, respectively.

A 1% chitosan solution of pH 6.8 was obtained by the adjustment of concentrated chitosan solution in acetic acid using a 10% ammonium phosphate solution followed by dilution in water. A reference solution of pH 6.8, lacking chitosan, was prepared similarly.

Fungal cultures were isolated from patients diagnosed clinically and mycologically as suffering from candidiasis. All fungal strains were identified and grown on standard Sabouraud medium. Isolated fungal strains were then used in the tests once it had been confirmed that they were resistant to neither clotrimazole nor nystatin, the lat-

ter being verified according to a standard disk diffusion method.

Fungal strains

Yeast-like: The following strains of fungi were used: *Candida albicans* (Ca) isolated from blood (Ca_1), stool (Ca_2), mucous membranes of the mouth (Ca_3–Ca_7), and mucous membranes of the vagina (Ca_8–Ca_{12}) as well as *C. crusei, C. glabrata* and *Rhodoturula* isolated from the buccal and vaginal mucosae.

Molds: The molds employed were as follows: *Aspergillus niger, Scopulariopsis brevicaulis, Penicillium* sp. and *Fusarium* sp. isolated from skin.

Dermatophytes: The dermatophytes were: *Microsporum canis, Trichophyton mentagrophytes, T. rubrum* and *Epidermophyton floccosum* isolated from skin.

Fungal cell suspensions

A *C. albicans* cell suspension was prepared by dissolving a 24 h culture of *C. albicans* in physiological NaCl solution and by the addition of *C. albicans* to the reference solution to achieve a given amount as determined by turbidimetry. Pretreatment of a *C. albicans* suspension lacking chitosan was carried out through shaking 1 ml of a *C. albicans* suspension containing 10^8 *C. albicans* cells.

Pretreatment of a *C. albicans* suspension containing chitosan was performed by shaking 1 ml of a solution containing 10^8 *C. albicans* cells (thereby diluting the *C. albicans* suspension) and 1 mg chitosan (thus diluting the reference solution).

After 30 min shaking at 37°C, the samples were centrifuged, the reference solution was washed three times and *C. albicans* was suspended in the solution. The possibility of an influence on the growth of *C. albicans* due to the reference solution was excluded by using the fungal growth test.

Cells

Human vaginal epithelial cells were collected, with informed consent, from several healthy volunteers by gently scraping the vaginal mucosae with a sterile wooden spatula, followed by immersion in physiological saline.

HeLa cells were obtained from the PZH Culture Collection (Warszawa).

Cell suspensions

Suspensions of both epithelial and HeLa cells were prepared by washing the cells in the reference solution, resulting in a given amount of cells; cell counts were established using a Bürker hemocytometer.

Pretreatment of epithelial cell suspensions in both the presence and absence of chitosan was carried out as described above for the *C. albicans* suspensions, with the exception of the number of cells which was 10^5 for epithelial cells.

Fungal growth tests

Addition of 200, 500 and 1000 mg chitosan or the corresponding amounts of the 1% chitosan solutions of pH 4.0 in acetic acid, pH 5.7 in lactic acid or pH 6.8 (reference solution) was made to a 1 l volume of Sabouraud medium. As control media, pure medium and media containing equivalent amounts of acetic acid, lactic acid or reference solution were made up. 2-day cultures of yeast-like cells and 5-day cultures of mold or dermatophyte cells were transferred to the media prepared as above.

The growth of yeast-like cell cultures which had been incubated at 37°C was determined after 2 days and that of mold and dermatophyte cell cultures incubated at 27°C after 7 days.

Fungal adherence test

The test consisted of the simultaneous shaking of 1 ml of the reference (control) solution and 1 ml of the test solution, the compositions of the solutions during the consecutively conducted tests being as follows:

1 Control – 10^8 *C. albicans* and 10^5 epithelial cells (from dilution of *C. albicans* and epithelial cell suspensions);
   Test – equal amounts of both cell types with the addition of 1 mg chitosan (from dilution of 1% chitosan solution of pH 6.8).
2 Control – 10^8 *C. albicans* cells (from dilution of pretreated *C. albicans* cell suspension) and

10^5 epithelial cells (from dilution of epithelial cell suspension);
Test – equal amounts of both cell types (from dilution of pretreated *C. albicans* cell suspension with chitosan and epithelial cell suspension).

3 Control – 10^8 *C. albicans* cells (from dilution of *C. albicans* cell suspension) and 10^5 epithelial cells (from dilution of pretreated epithelial cell suspension);
Test – equal amounts of both cell types (from dilution of *C. albicans* cell suspension and pretreated epithelial cell suspension with chitosan).

4 Control – 10^8 *C. albicans* and 10^5 HeLa cells (from dilution of *C. albicans* and HeLa cell suspensions);
Test– equal amounts of both cell types with the addition of 1 mg chitosan (from dilution of 1% chitosan solution of pH 6.8).

The above pairs of solutions were shaken for 30 min at 37°C, the suspensions being subsequently centrifuged and washed twice with reference solution. The number of fungal cells adhering per 50 epithelial cells or HeLa cells was counted under a microscope and the mean was then determined. This procedure was performed for each *C. albicans* strain. Each test was repeated three times. Significance of the results was evaluated according to the Wilcoxon test.

Results and Discussion

The fungal test in this work is routinely employed when cultivating pathogenic fungi and assessing the factors affecting their development. The test indicated that chitosan of 66% degree of deacetylation, introduced into the medium as a powder or as a solution of pH 4.0, 5.6 or 6.8, did not influence the growth of any of the 12 strains of pathogenic fungi isolated from material samples taken from patients showing clinical symptoms and signs of mycosis. Confirmation of these results was provided via the introduction of 10 g chitosan into the medium. Of course, a number of minor differences were evident in fungal growth between growth on pure medium and on medium with acetic acid, lactic acid or reference solution (used as control). However, such differences are insufficient to merit a detailed description being given here, since the corresponding growth intensities of the systems under examination (media with chitosan, or 1% chitosan solutions of pH 4.0 in acetic acid, pH 5.7 in lactic acid, or pH 6.8 (reference solution)) scarcely differed.

The present results are not in agreement with the data of Hirano and Nagao (1989) who reported that crab chitosan exerted an inhibitory effect on the growth of fungi generating plant infections and, to lower extent, on fungi pathogenic to humans. However, since the current study is aimed at a different goal, comparison with the previous paper will not be pursued. Nevertheless, the suggestion of Hirano and Nagao that depolymerised products of chitosan could be effective as inhibitors of fungal growth should be considered.

The fungal cell adherence test used here is often performed in vitro in order to monitor the

TABLE 1

Candida albicans adherence to human epithelial cells (tests 1–3) and HeLa cells (test 4)

Test no.	*C. albicans* (Ca) strains	Number of *C. albicans* cells attached to a single cell		$p <$
		Control ($\bar{x}$) (min–max)	Test ($\bar{x}$) (min–max)	
1	Ca_3–Ca_{12}	62.4 53.7–83.0	27.6 22.2–35.5	0.025
2	Ca_3–Ca_{12}	42.4 39.2–59.7	24.1 19.7–28.4	0.001
3	Ca_3–Ca_{12}	37.0 32.5–40.4	20.9 17.7–27.7	0.001
4	Ca_1–Ca_3	12.2 11.2–12.8	6.3 6.0–6.6	–

mechanisms of mycotic infection. Pretreatment of healthy host cells or of fungal cells with various additions enables one to identify the factors governing the process.

The use of HeLa cells as a model for the pathological modification of cells was intended not only to verify the data observed but also to devise experimental conditions approaching those occurring in vivo.

Data concerning the anti-cancer activity of chitosan have been reported (Sirica and Woodman, 1971; Knapczyk et al., 1989a). The results listed in Table 1 show that the addition of chitosan to a suspension of epithelial cells leads to a more than 2-fold reduction in the number of adhering *C. albicans* cells (test no. 1).

Pretreatment of fungal cells (test no. 2) or epithelial cells with chitosan (test no. 3) also retarded the process of adherence considerably. Good use has been made of such observations in a previous investigation by Segal et al. (1987). The latter authors suggested the use of a chitin soluble extract, isolated from *C. albicans* or from commercially available products, as a pharmaceutical for topical application in the prevention or alleviation of infections caused by yeasts, particularly for *Candida*.

The use of HeLa cells in our evaluation (test no. 4) confirmed our data. Although a statistical analysis was precluded due to the sparsity of data, a large difference between the surfaces of both cell types made a quantitative comparison of the data impossible.

Discrepancies found in previously reported studies (Lehrer et al., 1983, 1986; Segal et al., 1984) were successfully avoided as a result of performing the fungal adherence test using fungal cultures isolated from patients' symptomatic mycosis and fungi capable of growth while being susceptible to antimycotic drugs commonly in use. The combination of the tests allowed clarification of the role played by chitosan as an auxiliary component of formulations with antimycotic agents. Our findings indicated that chitosan does not influence fungal growth, but rather, it exerts a non-specific effect on both fungal and epithelial cells, thereby effectively inhibiting fungal adherence to host cells. Finally, chitosan prevents development of infections and thus enhances the efficacy of an applied drug. The simultaneous application of both tests may therefore be recommended as an economical, simple and widely available method for determining the efficacy of the components of formulations used in the treatment of mycotic infections.

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