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Original Article

In Vitro Activity of Pentamidine Isethionate against Trophozoite and Cyst of *Acanthamoeba*

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Abstract

Background: *Acanthamoebae* are a causative agent of *Acanthamoeba* keratitis (AK) in immunocompetent individuals. Since access to propamidine isethionate (Brolene®) as a first-line treatment has been limited in recent years, in the current study, we examined the effects of pentamidine isethionate against trophozoite and cyst forms of *Acanthamoeba*.

Methods: This experimental study was conducted in the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, during 2019-2020. Pentamidine isethionate at concentrations of 50, 100, 200, 400, 600, 800, and 1000 µM were tested against trophozoites and cyst stages of T4 genotype, at 24- and 48-hour incubation period, and the viability was determined by trypan blue staining. In addition, the cytotoxic effect of the drug was examined in *Vero* cells using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: The 50% inhibitory concentration (IC50) of pentamidine isethionate on trophozoite after 24 and 48h were 97.4 µM and 60.99 µM. These results on cyst after 24 and 48h were 470 µM and 175.5 µM, respectively. In MTT assay, the drug showed an inhibitory effect on *Vero* cell growth with IC50 values of 115.4 µM and 87.42 µM after 24h and 48h, respectively.

Conclusion: Pentamidine isethionate exhibited an inhibitory effect on trophozoite and cyst. Given that the trophozoicidal activity of the drug is in the safe dose, it could be suggested as an alternative in patients with AK; however, further investigation is needed in an animal model to confirm the data.

Introduction

Acanthamoebae are known to be a causative agent of *Acanthamoeba* keratitis (AK), often reporting from contact lens wearers (1), which are classified into 22 genotypes based on the 18s ribosomal RNA gene (2). The genotype T4 is the most prevalent isolate in clinical and environmental samples and the most virulent genotype with the highest potential of binding to host cells (3, 4).

Early diagnosis and AK's treatment are critical as the delay could lead to further complications such as blindness. It is well documented that the combination of biguanides and diamidines compounds is the pillar choice for AK's treatment to prevent drug resistance and persistent infection (5). Nevertheless, the eradication of *Acanthamoeba* from the affected tissue is challenging since during drug pressure and immune response trophozoites transform to cysts which is highly resistance to drugs. Moreover, disagreement still exists concerning the optimal approach of treatment (6). Some clinicians prefer to perform a penetrating keratoplasty to remove the cornea of the *Acanthamoeba*. In contrast, others choose medical therapy with topical propamidine isethionate (Brolene®), chlorhexidine digluconate, polyhexamethylene biguanide, miconazole, neosporin, corticosteroids, or more than one of these (7, 8). Diamidine derivatives and neosporin are also effective against amoeba in in-vitro assay (9-12).

Several reports have demonstrated that diamidine derivatives like Brolene® could be a drug of choice for the treatment of AK (13-16). However, most clinicians do not have access to this compound, as it is an over-the-counter drug sold only in Great Britain (17). In the group of aromatic diamidines, one of the alternative derivatives is pentamidine isethionate, which could be suggested as a substitute to Brolene® for treatment of AK.

Accordingly, in the present study, in vitro activity and cytotoxicity of the Pentamidine isethionate against the T4 genotype of *Acanthamoeba* were examined.

Materials and Methods

Acanthamoeba: strains and culture conditions

This experimental study was conducted in the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, during 2019-2020. *Acanthamoeba* T4 genotype (Accession number, KU936118.1) tested in the present study was previously isolated from a corneal scrape collected from a soft contact lens wearer affected with AK (17). For the preparation of trophozoite, the amoeba was grown axenically without shaking in 10 ml Pepton Yeast Glucose (PYG) medium, including 0.75% (w/v) protease peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose in T-25 tissue culture flasks (18). The axenic culture was kept in PYG at 30°C to gain the trophozoite. Also, for obtaining the mature cysts, the trophozoite was cultured on 1.5% non-nutrient agar (NNA) plates overlaid with 5µl of heat-killed *Escherichia coli* and incubated at 30 °C for three weeks.

Drug-susceptibility test on trophozoite stage

The trophozoite stage was collected and washed with phosphate buffer saline (PBS). Afterward, the number of trophozoites was determined by a hemocytometer. Experiments were carried out in 96-well microtiter plates (Nunc, NY, USA). Under sterile conditions at 37 °C. To evaluate the susceptibility of trophozoites to pentamidine isethionate (Sigma-Aldrich Ltd., Germany), each well was seeded with 2×10^5 cells/well of a trophozoite suspension; then, 100 µl of PYG medium containing

50, 100, 200, and 400 µM of the drug was added to all wells except untreated control wells that received 100 µl of the medium. The viability was determined after 24 and 48 h using trypan blue and a hemocytometer (19). Moreover, 0.02% (0.04 mM) chlorhexidine digluconate (CLX) (Sigma-Aldrich Ltd., Germany) was used against trophozoites and cysts as the reference drug (positive control) in all the experiments.

Drug-susceptibility test on cyst stage

The cysts were harvested and washed in phosphate-buffered saline (PBS), then 20×10^4 cysts/well were seeded in each microtube. The cysts were incubated with pentamidine isethionate (Sigma Aldrich, Germany) at concentrations of 50, 100, 200, 400, 600, 800 and 1000 µM except for untreated control tubes, which had only pure PBS and incubated at 37°C for 24 and 48 h. Following incubation, the viability of cysts was determined by staining with 0.4% trypan blue.

Cytotoxicity test

For cytotoxicity assays, the Vero cell is grown in DMEM (Gibco, Life Technologies GmbH, Germany) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U/ml penicillin, 100 µg/ml streptomycin. Cell proliferation was evaluated by performing a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. In brief, *Vero* cells (5×10^4 per well) were plated in 96-well plates (Nunc, NY, USA) and allowed to adhere 24 h. After washing, the cells were treated with increasing pentamidine isethionate (50, 100, 200, 400, 600, 800, and 1000 µM). After 24 and 48 h, 25 µl MTT (5 mg/ml MTT in DMEM) was added to the cells, and the mixture was incubated for an additional three h at 37°C. Subsequently, 125µl of DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) of each well was determined using Microplate ReadersELx800™ (BioTek, USA) at 570 nm. Cell viability in response to pentamidine isethionate administration was calculated using the following equation: Cell viability (%) = (OD treated / OD control) × 100.

Statistical analysis

All experiments were conducted at least two times, and the results are expressed as the mean ± standard deviations (SDs). Drug susceptibility assay was presented as IC50 (50% inhibitory concentration), determined using nonlinear regression analysis with the GraphPad Prism software version 8.02 (GraphPad Software Inc, La Jolla, CA). Repeated Measure two-way ANOVA (2-way R-M ANOVA) was performed on data obtained of bar graphs using GraphPad Prism. To determine statistical significance between groups, comparisons were made using Sidak's multiple comparisons Test. A *P*-value of 0.05 was accepted for statistical significance ($P<0.05$).

Results

Susceptibility of trophozoites

After 24 and 48 h of incubation, all pentamidine isethionate concentrations can inhibit trophozoite survival with different ranges. The results of eliminated trophozoites after 24 a d 48 h are shown in Table 1 and Fig. 1. The drug showed a statistically significant difference ($P<0.005$) compared to the non-treated control. The drug IC50 for inhibition of trophozoite survival after 24 h were 97.4 µM and 60.99 µM after 48 h, respectively.

Susceptibility of cysts

After 24 and 48 h of incubation, pentamidine isethionate in all concentrations could inhibit cyst survival but exhibited a different degree of activity. The results of the eliminated cyst are shown in Table 1 and Fig. 2. The drug showed a statistically significant difference ($P<0.005$) compared to the non-treated control. The IC50 for killing T4 after 24 h were 470 µM and 175.5 µM after 48 h.

Table 1: Concentration of pentamidine isethionate exhibited amoebistatic effects. (T4 cysts and trophozoites – 24 and 48 h)

Time	*Cyst*				*Trophozoite*			
	24 h		48 h		24 h		48 h	
(Conc. µM)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	98.667	0.471	98.667	0.471	98.66	0.57	98.66	0.57
50	91.333	1.247	89.333	0.471	91.33	1.52	37.33	4.72
100	88.333	0.471	77.333	0.943	46.66	4.16	27	2.64
200	80.000	1.633	69.000	0.816	5.33	2.51	0	0
400	66.667	1.700	51.333	4.989	0	0	0	0
600	33.667	3.091	33.000	2.160				
800	3.333	0.471	1.667	0.471				
1000	0.000	0.000	0.000	0.000				

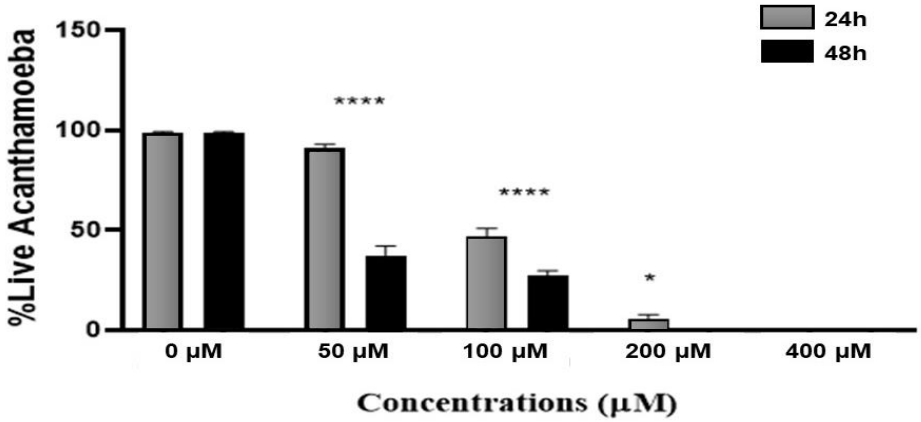

Fig. 1: Pentamidine isethionate inhibitory effects on T4 Trophozoites over 24 and 48 h. 0: non-treated control, all results are expressed as a total percentage of viable cells with mean ± standard deviation (SD) of three independent determinations and analyzed using the two-way Repeated Measure analysis of variance (R-M ANOVA) and Sidak's Multiple comparisons Test at a significance level of $P < 0.05$ (indicated by *< 0.05, **< 0.01, ***< 0.001 and ****< 0.0001)

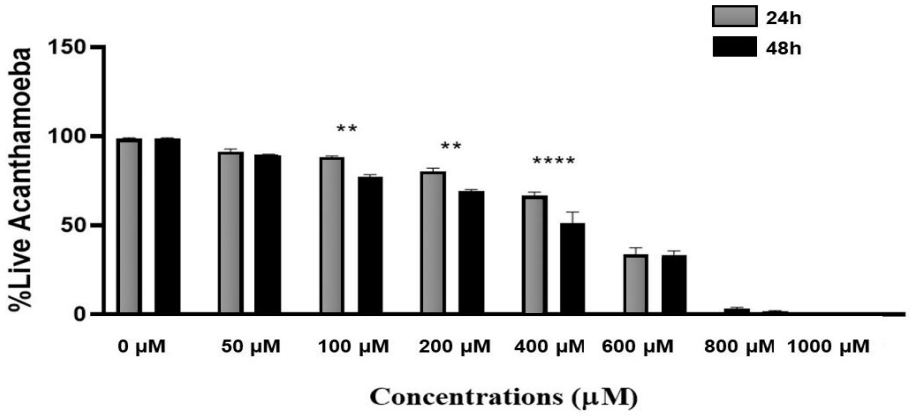

Fig. 2: Pentamidine isethionate inhibitory effects on T4 Cysts over 24 and 48 h. 0: non-treated control, all results are expressed as a total percentage of viable cells with mean ± standard deviation (SD) of three independent determinations and analyzed using the two-way Repeated Measure analysis of variance (R-M ANOVA) and Sidak's Multiple comparisons Test at a significance level of $p < 0.05$ (indicated by *< 0.05, **< 0.01, ***< 0.001 and ****< 0.0001)

Effect of pentamidine on Vero cell proliferation and apoptosis

The administration of pentamidine (50, 100, 200, 400, 600, 800, and 1000 μM) to Vero cells caused a significant concentration-dependent decrease in cell viability (IC50 115.4 μM and 87.42 μM after 24 h and 48 h, respectively) compared with the unstimulated cells (assumed 100% viability) (Fig. 3).

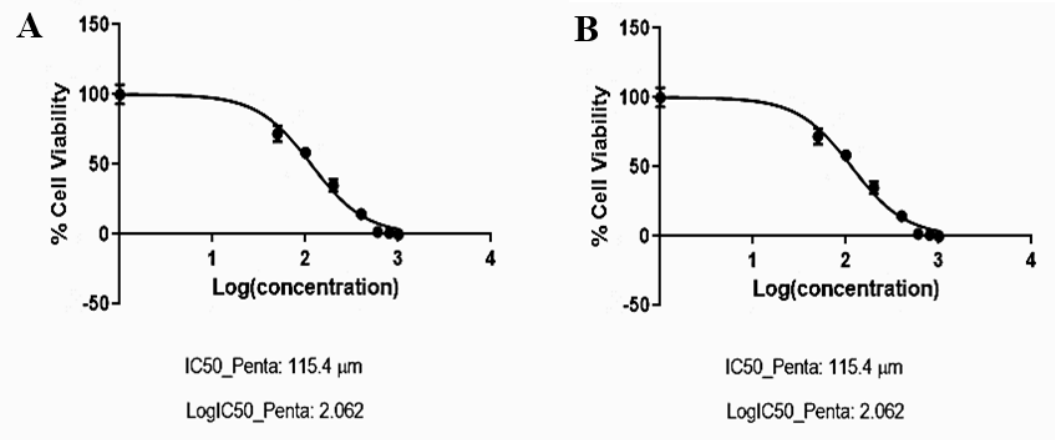

Fig. 3: The cytotoxicity effect of pentamidine isethionate on *Vero* cell lines over 24, 48 h using MTT assay :(A) 24h, (B) 48h. X-axis: Drug concentrations (log) from 50 to 1000 μM and Y-axis: the normalized cell viability percentage. Values are mean ± SD of three independent experiments

Discussion

Treatment of AK is impaired due to parasitic resistance. Since topical propamidine isethionate (Brolene®) is currently the drug of choice in Europe, we sought to determine whether pentamidine isethionate might be equally effective, as the drug is more available worldwide to ophthalmologists. This study investigated amoebicidal activity of this compound against T4 genotype in vitro and its cytotoxic activity on Vero cells. All tested concentrations of drug could inhibit cyst and trophozoite survival but exhibited a different degree of activity.

In an earlier study (20), different species of *Acanthamoeba* exhibited discrepancies in susceptibility against pentamidine and propamidine drugs. Propamidine (>1,000 μg/m1) was less effective than pentamidine (>125 μg /ml) against *A. castellanii*, although equivalent potency (>250 μg/ml) was detected against *A. polyphaga*. On the other hand, propamidine (>31.25 μg/m1) was slightly more efficient than pentamidine (>62.5 μg / ml) against *A. hatchetti*. Both drugs were entirely nontoxic in the in vitro test; however, propamidine at the effective dose was considerably more toxic, representing a lower therapeutic index of propamidine (20).

Huang et al. in the first report on AK cases treated with pentamidine isethionate reported that the average duration of treatment was 9.8±3.5 days, with no serious side effects. Visual acuity improved from 1.41logMAR±1.00logMAR to 0.19logMAR±0.34logMAR. Nevertheless, they concluded that a larger prospective population-based comparative study is required to confirm the efficacy of the drug (21).

Both free and liposomal pentamidine isethionate inhibited *Acanthamoeba* encystation (22). When incubated alone, there was approximately 50% encystment. At the concentration of 10 μg ml^{-1}, this compound reduced amoeba encystment to 12.5%±3.5. Although there were minor differences between drug alone,

ergosterol-drug, and cholesterol-drug at 2 µg ml-1, these differences were not significant.

More studies need to be done in this manner. Most of the drug used as an eye drop is toxic like chlorhexidine; however, circulation of tears in the ocular system and small drop sizes help prevent their toxic effects (22).

Conclusion

As pentamidine's cytotoxicity is higher than its IC50, the drug is toxic. Therefore, the use of a carrier that encapsulates the drug in the biological structure could make possible the in vitro usage of pentamidine. Further investigation is suggested on the liposomal and other nanocarriers forms against various *Acanthamoeba* isolates of different genotypes to reduce the toxicity.

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Conflict of interest

The authors declare that they have no conflict of interest.

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