

Anti-Acanthamoeba Potential of Valproate Sodium; an In Vitro Study

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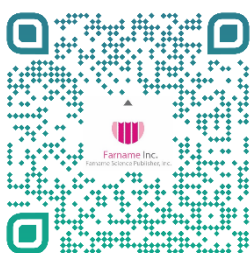
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ABSTRACT

Background & Objective: Conventional treatment of Acanthamoeba typically involves a combination drug strategy, but its efficacy in clinical settings remains incomplete. Evaluating the therapeutic potential of existing drugs is a way used to introduce effective treatments for infectious agents. This study aimed to assess the in vitro anti-Acanthamoeba effect of valproate (VPA).

Materials & Methods: An experimental study was conducted using Acanthamoeba cysts belonging to the T4 and T5 genotypes. Cysts collected from the culture medium were exposed to gentamicin, polymyxin, and three different concentrations of VPA for varying durations (1, 4, 6, and 24 hours). The treated cysts were stained with trypan blue, and the percentage of growth inhibition was calculated. Additionally, the viability of treated cysts was assessed by culturing them on non-nutrient agar plates for one month.

Results: The Acanthamoeba cysts of T4 and T5 genotypes showed susceptibility to VPA. The minimal cysticidal concentration (MCC) of VPA for maximum growth inhibition in both single and combination drug assays were 100 and 3 mg/ml, for durations of 24 and 4 hours, respectively. The growth inhibition observed in the groups exposed to gentamicin and polymyxin differed significantly from the growth inhibition in the group treated with ≥ 100 mg/ml VPA ($P < 0.05$).

Conclusion: VPA enhances the effects of gentamicin and polymyxin on Acanthamoeba. Combining a low concentration of VPA (≥ 3 mg/ml) with gentamicin and polymyxin increases the potency and speed of action of these antibiotics.

Keywords: Acanthamoeba, Gentamicin, Polymyxin, Valproate, Single Drug Strategy, Combination Drug Strategy



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Introduction

Unsuccessful treatment of conventional drugs for certain infections necessitates the development of new alternative drugs or the evaluation of different drug classes for effective treatment. Acanthamoebiasis is an infection caused by the amoeba "Acanthamoeba" that specifically targets the nervous and ocular systems (1).

The prevalence of this parasite is extensive, as it has been detected in water and soil sources across numerous regions worldwide (1). Furthermore, reports indicate the contamination of equipment and devices with the parasite in various hospital wards (2). Acanthamoeba cysts play a pivotal role in the transmission and dissemination of this parasite (1).

The in vitro evaluation of various chemical drugs and herbal derivatives has been conducted to assess their anti-Acanthamoeba effect, and several have shown promising results (3-5).

Regrettably, the therapeutic effectiveness of these drugs has not proven to be satisfactory in both in vivo studies and clinical applications (3). Additionally, even in

instances where successful clinical treatment has been documented, the duration of treatment has been considerably prolonged (3). Consequently, it appears inevitable that new chemical drugs or alternative drug classes, which are both more effective and safer in the clinical treatment of Acanthamoeba infections, must be explored (3).

Gentamicin and polymyxin are commonly utilized as topical treatments for acanthamoeba eye infections (6, 7). However, in vitro, evaluations of the cysticidal efficacy of these drugs have revealed that while they inhibit the growth of cysts, they have not demonstrated complete effectiveness in treating Acanthamoeba keratitis (3, 6, 7).

Valproic sodium or valproate (VPA) is extensively prescribed for the treatment of generalized or partial seizures (8). It has been well-documented that this drug possesses antimicrobial activity (9). One mechanism involved in regulating various biological processes, including proliferation in eukaryotes, is histone deacetylases. This mechanism plays a crucial role in

modulating chromatin remodeling through complex processes (10). The potential of VPA as an inhibitor of histone deacetylase 1 in *Plasmodium falciparum* has been elucidated (10). Furthermore, the antiproliferative and antiencystation effects of histone deacetylase on *Acanthamoeba* have been confirmed (11).

Therefore, this study aimed to assess the cysticidal efficacy of valproate against two pathogenic strains of *Acanthamoeba* (Iran isolates). Additionally, the study investigated the effects of combining VPA with antibiotics. Hence, the anti-*Acanthamoeba* effect of VPA was compared to that of gentamicin and polymyxin, which served as reference drugs in this study.

Materials and Methods

This experimental study was approved by the Ethics Committee of Arak University of Medical Sciences with the reference number IR.ARAKMU.REC.1400.147.

Preparation of parasite

Two strains of *Acanthamoeba*, namely MG066681 (T4 genotype) and MG298789 (T5 genotype), obtained from the parasitology laboratory at Arak University of Medical Sciences, were utilized in this study (12).

The isolates were cultured on non-nutrient agar plates (NNA) coated with killed *E. coli* at a temperature of 28°C. Cysts were harvested from the culture medium and washed thrice with phosphate-buffered saline (PBS). The viable and nonviable cysts were then counted using trypan blue staining (unstained cysts indicating viability, stained cysts indicating nonviability) and a hemocytometer. A parasitic suspension with at least 90% viable cysts was prepared for the study. Finally, the cyst concentration in the parasitic suspension was standardized using a hemocytometer (2×10^5 cysts).

Drugs

Three therapeutic drugs were tested individually: gentamicin (3 mg/ml) (Sina Darou, Tehran, Iran), polymyxin B (10000 U/ml) (Sina Darou, Tehran, Iran), and Valproate sodium (VPA, with concentrations of 3, 50, 100, and 200 mg/mL) (Darou Pakhsh Pharma Chem. Co., Tehran, Iran). Additionally, three combination therapies were tested: gentamicin in combination with 3, 50, 100, and 200 mg/mL VPA and polymyxin in combination with 3, 50, 100, and 200 mg/mL VPA. Phosphate buffer solution (PBS) was used as the control (untreated).

Experiments

200 µL of the parasitic suspension containing two *Acanthamoeba* strains was mixed with 200 µL of PBS, gentamicin, polymyxin, and four different concentrations of VPA (final concentrations of VPA: 3, 50, 100, and 200 mg/ml) in sterile microtubes. The mixture was then incubated for 1, 4, 6, and 24 hours at 37°C. After each exposure period, the cysts were washed three times with PBS to eliminate any remaining drugs. The same

procedure was followed for the combination therapy drugs.

The washed cysts were resuspended in 100 µL of PBS. A portion of each experiment was allocated for the cyst viability assay, while the remaining portion was used to investigate viable and nonviable cysts and calculate the percentage of eliminated cysts (or percent growth inhibition) using the Trypan blue exclusion assay (5, 13). Each strain was subjected to six repetitions of the experiments.

Cyst viability assay

Cyst viability was assessed using the following procedure. Thirty (30) µL of the drug-exposed cyst suspension (obtained from the previous step after being washed three times with PBS) was transferred to NNA medium plates coated with killed *E. coli* and incubated at 28°C. These plates were observed daily under a microscope for a duration of one month.

Calculation of percent of growth inhibition (or % eliminated cysts)

In summary, the cyst suspension was mixed with trypan blue at a 1:1 ratio. The resulting mixture was loaded into a hemocytometer, and the number of viable and nonviable cysts were counted under a light microscope. The calculation was carried out using the following method (13).

$$\% \text{ viable cysts} = \frac{\text{Total number of viable cysts per ml}}{\text{Total number of cysts per ml}} \times 100$$

$$\% \text{ growth inhibition (or \% eliminated cyst)} = 100 - \% \text{ viable cysts}$$

Data analysis

The data analysis was conducted using SPSS software (version 23, SPSS/PC Inc., Chicago, IL, USA) and Excel (2016). The results were presented as mean \pm SD and as percentages of eliminated cysts (or percent growth inhibition). Differences between the groups were analyzed using the Two-way ANOVA test. Statistical significance was defined as $P < 0.05$.

Results

The effects of gentamicin and polymyxin on *Acanthamoeba* cysts were compared to the effects of different concentrations of VPA on two genotypes of *Acanthamoeba* cysts (Table 1 & Figure 1). The results demonstrated that the percentage of growth inhibition in both genotypes of the parasite increased with longer exposure times and higher drug concentrations of VPA. Cysts of both *Acanthamoeba* genotypes were completely eliminated at concentrations of 100 and 200 mg/ml VPA, with respective durations of 24 and 6 hours for complete eradication. Furthermore, there was no significant difference in the effect of VPA on the two *Acanthamoeba* genotypes ($P > 0.05$). Statistical analysis revealed that the percentage of growth inhibition in all groups treated with gentamicin, polymyxin, and

different concentrations of VPA (except the group exposed to 3 mg/ml VPA) significantly differed from the growth inhibition observed in the untreated control (PBS) ($P < 0.0001$). Additionally, the growth inhibition between the groups exposed to gentamicin and polymyxin significantly differed from the growth inhibition in the group exposed to ≥ 100 mg/ml VPA ($P < 0.05$) (Figure 1: A & C).

The results of this study also demonstrated that the combination of VPA with gentamicin and polymyxin

increased the effectiveness of VPA. Specifically, the concentration of 3 mg/ml VPA, when used in combination with each of the two mentioned drugs, completely eradicated cysts of both genotypes of the parasite within 4 hours. Statistical analysis revealed that the viability rate of this parasite in all groups treated with combinations of drugs significantly differed from the viability rate of the parasite in the untreated control (PBS) ($P < 0.05$) (Figure 1: B & D).

Table 1. Growth inhibition percent of *Acanthamoeba* (or Eliminated cysts) treated with Gentamicin, Polymyxin and different concentration of VPA

<i>Acanthamoeba</i> genotype	Exposure time (h)	Percentage of growth inhibition (%)						
		PBS (control)	Gentamicin	Polymyxin	VPA 3 mg/ml	VPA 50 mg/ml	VPA 100 mg/ml	VPA 200 mg/ml
T4	1	1.8	53.5	34.3	2.83	52.8	86.3	91.3
	4	1.8	86.7	55.2	2.83	80.0	92.8	97.3
	6	2	98.8	86.7	3.83	92.3	99.3	100
	24	2	100	100	4.7	99.3	100	100
T5	1	1.8	53.3	34	2.7	52.8	86.3	91.3
	4	2	86.7	54.7	2.7	80.7	92.8	97.8
	6	2	98.8	89.7	3.5	92.3	99.2	100
	24	3	100	100	4.7	99.3	100	100

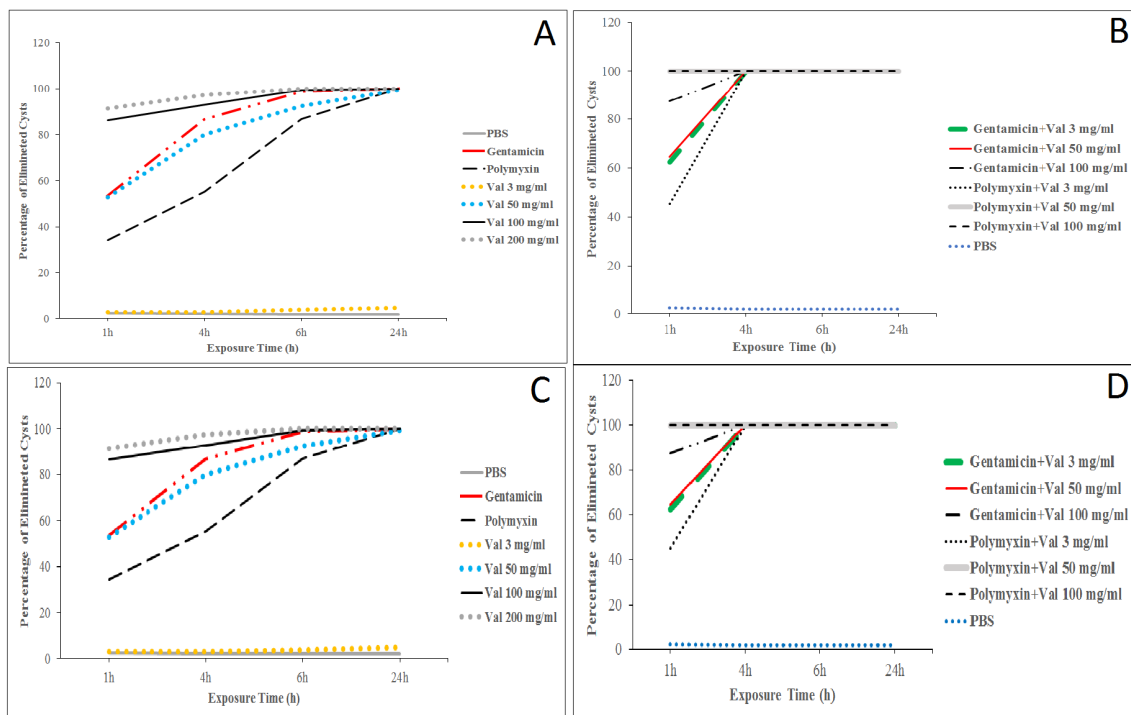


Figure 1. Effects of single and combination drugs on the percentage of growth inhibition (or eliminated cysts) of *Acanthamoeba* cysts. A & B: T4 genotype of *Acanthamoeba* treated with single and combination drugs, respectively. C & D: T5 genotype of *Acanthamoeba* treated with single and combination drugs, respectively.

In the present study, treated cysts from all groups were transferred to NNA medium for cyst viability assay. This assay validated the findings of the percentage of growth inhibition. Cysts of T4 and T5 genotypes lost their reproductive ability after 24 hours of exposure to single drugs and did not exhibit growth or multiplication in the NNA culture medium.

Furthermore, the findings of this study indicated that the Minimal Cysticidal Concentration (MCC) of VPA as a single drug was 100 mg/ml, resulting in the

complete eradication of all cysts of T4 and T5 genotypes after 24 hours (Table 1). The MCC values for the combination drugs were as follows: the MCC of VPA in combination with gentamicin and polymyxin was 3 mg/ml, leading to the complete elimination of all cysts of T4 and T5 genotypes within 4 hours (Table 2).

The morphological changes observed after a 24-hour incubation of cysts with each drug are presented in Figure 2.

Table 2. Growth inhibition percent of *Acanthamoeba* (or Eliminated cysts) treated with Gentamicin and Polymyxin plus different concentrations of VPA

<i>Acanthamoeba</i> genotype	Exposure time (h)	Percentage of growth inhibition (%)						
		PBS (control)	Gentamicin +VPA (3 mg/ml)	Gentamicin + VPA (50 mg/ml)	Gentamicin + VPA (100 mg/ml)	Polymyxin + VPA (3 mg/ml)	Polymyxin + VPA (50 mg/ml)	Polymyxin + VPA (100 mg/ml)
T4	1	1.8	62.5	64.5	87.3	45.3	100	100
	4	1.8	100	100	100	100	100	100
	6	2	100	100	100	100	100	100
	24	2	100	100	100	100	100	100
T5	1	1.8	61.8	64.7	86.9	45.2	100	100
	4	2	100	100	100	100	100	100
	6	2	100	100	100	100	100	100
	24	3	100	100	100	100	100	100

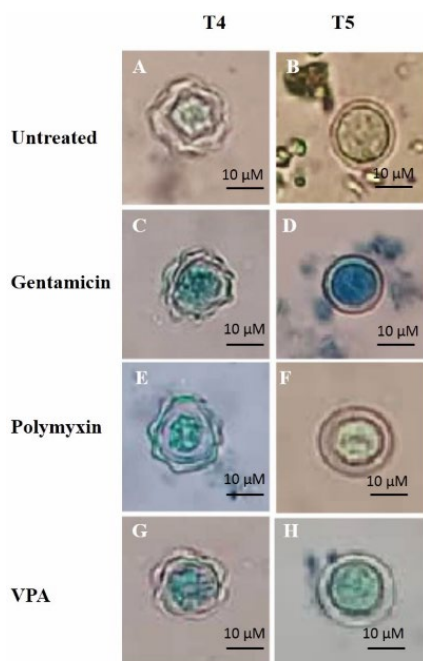


Figure 2. Effect of each drug on *Acanthamoeba* cysts of two genotypes after 24 hours of exposure time, observed under light microscopy ($\times 40$). A & B: Untreated cysts, C & D: Treated with Gentamicin, E & F: Treated with Polymyxin, G & H: Treated with 3 mg/ml VPA

Discussion

In this study, we assessed the efficacy of VPA, either alone or in combination with gentamicin and polymyxin, against *Acanthamoeba*. Currently, two recent medications are commonly used as anti-*Acanthamoeba* drugs in clinical cases. Our results confirm the cysticidal effect of different concentrations of VPA, regardless of whether it was used as a single drug or in combination. However, when used as a single drug, complete parasite mortality was observed only at high concentrations (≥ 100 mg/ml) of VPA. On the other hand, when combined with gentamicin and polymyxin, complete parasite mortality was achieved at a much lower concentration of VPA (3 mg/ml) (Tables 1 and 2). Importantly, the effectiveness of VPA, either alone or in combination, was not influenced by the genotype of the parasite.

The ineffectiveness of traditional drugs in treating infections caused by this parasite has prompted the investigation of the anti-*Acanthamoeba* activity of various chemicals, herbal compounds, and even non-specific drugs (3, 4).

This approach may unveil a novel application for such drugs, which could potentially be safer and more

effective than the conventional anti-Acanthamoeba medications.

Acanthamoeba, known for causing severe infections, poses a challenge in treatment due to its resistance to numerous drugs, primarily due to its cystic stage (14). Therefore, it is crucial to assess the cysticidal effect of various therapeutic drugs *in vitro* as a prerequisite for conducting subsequent *in vivo* therapeutic studies on acanthamoebiasis, particularly Acanthamoeba keratitis (15).

Currently, drug therapy serves as the foundation for the medical management of Acanthamoebiasis, with a particular focus on employing combination strategies to harness the synergistic effects of drugs (3).

VPA, a fatty acid, was synthesized approximately 140 years ago. However, its application as an anticonvulsant drug started around 55 years ago in various countries (16, 17). Over the past two decades, extensive research has been conducted on the anticancer and antimicrobial properties of VPA, including its effectiveness against bacterial and fungal infections, and these findings have been validated (8, 9, 18, 19).

Research has also been conducted on the antiparasitic properties of VPA. Fond *et al.* (2014) conducted a study comparing the *in vitro* anti-toxoplasmic effects of various drugs, including VPA. However, the findings indicated that valproate did not demonstrate any inhibitory effect on the growth of *Toxoplasma* (20).

Elbadawi *et al.* (2015) conducted a study that examined and confirmed the potential of VPA as an inhibitor of histone deacetylase 1 in *Plasmodium falciparum* using an *in silico* approach (10).

Following the confirmation of the antiparasitic potential of VPA in previous studies, the results of the present study also confirmed its anti-Acanthamoeba activity *in vitro*. The minimum concentration of VPA used in this study was 3 mg/ml, which was determined based on the results of a pilot study. The following are the noteworthy results of this study.

Application of VPA as a single drug at a concentration of ≥ 50 mg/ml significantly inhibits the growth of Acanthamoeba, resulting in complete cyst death after 24 hours of exposure. Lower concentrations of VPA within the same time frame do not have a significant effect on inhibiting the growth of Acanthamoeba. A comparison of the growth inhibition of Acanthamoeba in a single-drug assay using equal concentrations of gentamicin, polymyxin, and VPA demonstrates that gentamicin exhibits higher growth inhibition activity (Table 1). Both gentamicin and polymyxin exhibit maximum growth inhibition rates (100%) after 24 hours, whereas VPA shows a growth inhibition rate of 4.7% at the same concentration and exposure time. Previous studies have assessed the minimal cysticidal concentration (MCC) of gentamicin for Acanthamoeba. For instance, an *in vitro* study

reported that a concentration of 3 mg/ml of gentamicin completely inhibited the growth of Acanthamoeba and eliminated cysts within one hour (6). In contrast, the results of the current study, conducted under similar conditions (parasite genotype and drug concentration), showed a much lower rate of growth inhibition. Specifically, complete growth inhibition of the parasite was observed after 24 hours in the present study (Table 1 and Fig. 1). Summarizing previous studies; it has been found that concentrations ranging from 0.03 to 20 mg/ml of gentamicin are effective against various genotypes and strains of Acanthamoeba at temperatures of 30 and 37 °C for durations ranging from 1 to 48 hours (6, 7, 21, 22).

Exploring the underlying reasons for these differences is a topic worthy of consideration, and it is possible that they are associated with the physicochemical characteristics of the drug.

In a study, the cysticidal effect of natamycin (a commonly used drug for keratitis) was compared with extracts from green and black tea. The findings confirmed that the tea extracts exhibited a faster and more pronounced effect compared to natamycin. Moreover, the mortality rate of Acanthamoeba when exposed to various concentrations of tea extracts (both black and green) reached 30% within 24 hours (23).

However, comparing these results with the findings of the current study confirms that the effect of tea extracts is inferior to that of VPA.

The Azoles, including imidazole and triazole, exhibit anti-Acanthamoeba activity (24).

This drug class demonstrates cysticidal effects on Acanthamoeba at various concentrations. However, the impact of azoles on Acanthamoeba is comparatively slower and less pronounced when compared to VPA.

The findings of a recent study have corroborated the synergistic anti-Acanthamoeba effect of chlorhexidine and *Garcinia mangostana* extract on Acanthamoeba cysts (25).

In this study, the maximum growth inhibition of Acanthamoeba cysts was observed at 72 hours, which is slower compared to the effect of valproate in the current study. However, the drug concentration used in this study was lower than the concentration of valproate.

Due to the water solubility of VPA, it is convenient to investigate its synergistic effects or perform combination drug assays with other drugs (26).

In this study, combination drug assays of VPA with gentamicin or polymyxin were conducted. The results demonstrated that the combination of 3 mg/ml VPA with gentamicin or polymyxin enhanced their effects. The growth inhibition of Acanthamoeba cysts significantly increased within the first hour of exposure and reached its maximum (100%) by the fourth hour. For instance, when comparing the growth inhibition of

parasites in single-drug assays (gentamicin) and combination-drug assays (gentamicin with VPA), the growth inhibition of *Acanthamoeba* increased from 53.5% to 62.5% (Tables 1 and 2). Similar results were observed for polymyxin. Therefore, VPA appears to accelerate the effects of gentamicin and polymyxin on *Acanthamoeba*.

Given the confirmed use of VPA in humans, there is potential to develop new combination therapies for the treatment of *Acanthamoeba* keratitis in humans through further *in vitro* and *in vivo* studies. Additionally, exploring the use of injectable forms of VPA for the treatment of *Acanthamoeba* encephalitis could be considered. Considering VPA's role as a histone deacetylase inhibitor, its effect on *Acanthamoeba* may be dependent on this mechanism. Therefore, more research can be conducted in this area. Furthermore, investigating the anti-*Acanthamoeba* effect of VPA-like compounds and re-evaluating its efficacy through structural modifications of VPA is suggested.

Conclusion

This study confirms that VPA has anti-*Acanthamoeba* potential. Also, based on the results of this study, it seems that a low concentration of VPA (≥ 3 mg/ml) in combination with gentamicin and polymyxin increases the potency and speed of action of these antibiotics. It should be noted that high concentrations of VPA (≥ 100 mg/ml) as a single drug can lead to the complete death of *Acanthamoeba*. To date, no study has been reported on the anti-*Acanthamoeba* activity of VPA, and more research is needed to achieve more accurate and reliable results.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Study concept and design, Reza Rahimi and Zahra Eslamirad; Parasite preparation, Reza Hajihosseini; Performing the experiments, Zahra Eslamirad and Reza Hajihosseini; Data analysis, Reza Rahimi; Drafting of the manuscript, Zahra Eslamirad and Reza

Rahimi and Reza Hajihosseini; Revising the manuscript, Zahra Eslamirad.

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