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Effect of povidone iodine, chlorhexidine digluconate and toyocamycin on amphizoic amoebic strains, infectious agents of *Acanthamoeba* keratitis – a growing threat to human health worldwide

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Abstract

Introduction. Free-living amoebae, ubiquitous in outer environments, in predisposing circumstances may exist as parasites, infectious agents of Acanthamoeba keratitis. In recent decades, the vision-threatening corneal infection is a growing human health threat worldwide, including Poland. The applied therapy is often ineffective due to diagnostic mistakes, various pathogenicity of Acanthamoeba strains and high resistance of cysts to drugs; many agents with possible anti-amoebic activity are still being tested. In the presented study, selected chemicals are investigated in terms of their *in vitro* effect on corneal and environmental Acanthamoeba strains.

Materials and method. Samples of a corneal isolate from a patient with severe Acanthamoeba keratitis,of assessed on the basis of genotype associations of 18S rRNA and the type strain, Acanthamoeba castellanii Neff cultivated in bacteria-free condition, were exposed to povidone iodine, chlorhexidine digluconate or toyocamycin. *In vitro* population dynamics of the strains were monitored and compared to those of control cultures.

Results. All chemicals showed anti-amoebic effects with different degrees of effectiveness. Significant differences were observed in the *in vitro* population dynamics, and the morpho-physiological status of A. castellanii Neff T4 and corneal strains determined as A. polyphaga T4 genotype, exposed to povidone iodine or toyocamycin, in comparison with chlorhexidine taken as reference.

Conclusions. Time-dependent amoebstatic *in vitro* effects were demonstrated for all agents, in particular, the results of assays with povidone iodine are promising. No significant stimulation of encystation appeared; however, as cysticidal efficacy of chemicals is expected, complementary research is needed on different Acanthamoeba strains with modified agent concentrations and method application.

Key words

in vitro, Acanthamoeba Keratitis, A. polyphaga, T4 genotype, A. castellanii Neff, povidone iodine, chlorhexidine digluconate, toyocamycin effects

INTRODUCTION

Free-living amoebae (FLA) belonging to the genus *Acanthamoeba* are exozoic organisms widely distributed in various parts of the world, including Poland [1–16]. The protists exist as active feeding trophozoite forms that under adverse conditions transform into double-walled dormant cysts. The amoebae complete their life cycles in outer natural and man-made environments. They occur in various soil and aquatic habitats: in the soil, air, water (including tap and chlorinated water), drinking water systems, bottled mineral water, thermal recreational waters, and swimming pools; they are also often isolated from vegetables and fruits. The protists are found in dust and sewage, in air-conditioning systems, and humidifiers; they are also detected in health facilities, on surfaces of equipment, contact lenses and lens boxes, on accessories, surgical instruments, in dialyzers and in dental irrigation units. Moreover, the amoebae may also act as vehicles/vectors for fungi, protists, viruses and/or bacteria, including species pathogenic for humans from the genera *Escherichia*, *Chlamydia*, *Pseudomonas*, *Legionella*, and *Candida,* which may survive and even proliferate within the *Acanthamoeba* trophozoites [15, 17, 18].

In favourable conditions, some species of the amoebae may enter and colonize various animals, including humans, and exist as endozoic organisms – facultative parasites [4,

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6, 9–18]. For this reason, the FLA are considered to be amphizoic protists because they are able to exist in two different modes: as free-living exozoic amoebae and parasitic endozoic organisms causing pathogenic effects.

Some *Acanthamoeba* species may cause serious diseases in humans: rare but life-threatening granulomatous amoebic encephalitis, an opportunistic disease that develops in immuno-compromised individuals [2, 3, 4, 17, 19], and the non-opportunistic, progressive, sight-threatening eye disease, *Acanthamoebic* keratitis (AK) in immuno-competent patients [4, 15, 16, 17, 20, 21, 22, 23].

The amoebic infection of the human cornea was first reported by Nagington et al. [20]. Disease symptoms include tearing, redness, severe pain, photophobia, visual impairment, corneal ulcerations, and epithelial defects. Up to 90% of AK cases have been reported in contact lens wearers and related to improper contact lens hygiene. It is emphasized in the literature, and also confirmed by the experience of the authors of the current study, that AK is diagnosed with increasing frequency, including in Poland, along with the spread of the use of contact lenses [8, 15, 16, 20–29]. Important risk factors predisposing to *Acanthamoeba* infections in humans, apart from wearing contact lenses, are damage to the corneal epithelial cells, eye surgery, and exposure of the eye to water containing amphizoic amoeba trophozoites and/or cysts [15, 17, 21, 28, 30].

In recent decades, vision-threatening AK corneal infection has become a growing threat to human health worldwide. Applied therapy is often disappointing due to misdiagnostics, different pathogenicity of *Acanthamoeba* species and extreme resistance of *Acanthamoeba* cysts to disinfectants and drugs [8, 15, 16, 17, 23, 30, 31, 32]. For this reason, during the years of multicentre research, conducted also by the authors of the current study, different agents with possible activity against various *Acanthamoeba* strains are still being tested.

OBJECTIVE

In the current research, *in vitro* effects of selected chemicals were investigated on pathogenic *Acanthamoeba* isolate acquired and identified from complicated infectious keratitis, and on the environmental strain. Influence of the agents on the amoebic population dynamics *in vitro* was also examined, compared and evaluated. The study was performed in accordance with the tenets of the Declaration of Helsinki.

MATERIALS AND METHOD

***Acanthamoeba* strains investigated.** The strains included in this study were two different *Acanthamoeba* species, environmental and pathogenic, cultivated *in vitro* in the laboratory of the Department of Medical Biology, Medical University of Warsaw, Poland.

The material of the ocular isolate was derived from a non-wearer of contact lenses with a history of swimming in a lake, referred to hospital from another centre following previous misdiagnosis and unsuccessful treatment with antibacterial and antifungal medications.

Clinically, corneal ulcer and active epithelial inflammation in the course of complicated, severe *Acanthamoeba* keratitis were detected by non-invasive methods: the slit-lamp and *in vivo* confocal microscopy.

The material acquired from corneal scrapings was examined with a light microscope to visualize cysts or/and trophozoites of the amoebae. Samples of the corneal strain were cultured *in vitro* in sterile 15.0 ml tubes. Cultivation of this amoebic strain was performed under bacteria-free conditions in BSC medium (composed of Bacto Casitone, Difco, dissolved in water, enriched with 10% calf serum from Wytwórnia Surowic i Szczepionek, Lublin, with the addition of an aqueous solution of antibiotics: streptomycinum, penicilinum), as in earlier research [19, 23, 27, 28], at 26°C and regularly sub-cultured twice a month.

Specific identification of the corneal strain was performed using molecular techniques based on genotype associations of the 18S rRNA gene sequence. An *Acanthamoeba* specific PCR following the protocol established by Schroeder et al. [33] was applied. Direct sequencing of the obtained PCR product was performed. The sequence was analyzed using GeneStudio Pro Software, and compared with data from the GenBank to establish the genotype of the isolate.

The environmental type strain *A. castellanii* Neff (classified earlier as a species belonging to *Acanthamoeba* group II), defined by Schroeder et al. [33] for ATCC 30010 *A. castellanii* Neff T4 genotype, cultivated and monitored for years in the same BSC growth medium in the Department of Medical Biology laboratory, Medical University of Warsaw, Poland.

Acanthamoeba populations investigated were sub-cultured twice a month and their samples regularly observed for *in vitro* growth under direct light microscope. A range of amoeba number of two or three counts with the use of Bürker's hemocytometer calculated for 1 ml of culture medium was assessed. Before the exposure to chemicals, the dynamics of developmental forms of the amoebic strains was monitored during each sub-culturing and in the exponential growth phase.

Chemicals tested for effects on *Acanthamoeba* strains. Three compounds were tested for their potential *in vitro* activity against the *Acanthamoeba* strains investigated: povidone iodine PI, chlorhexidine digluconate CXD, and the adenosine analogue toyocamycin Toyo.

The monitoring of *in vitro* dynamics of amoebic strains was conducted during each sub-culturing and in subsequent growth phase, at the beginning, in the log phase; before the exposure to chemicals the overall amoebae count was in a range from $2–5 \times 10^4$/1 ml. Seven days after current sub-culturing, in the exponential phase of the cultivation cycle, samples of cultures of *Acanthamoeba* corneal strain and type strain *A. castellanii* Neff T4 genotype were exposed to the tested chemicals and compared in terms of their *in vitro* susceptibility to these agents. All experiments were performed in sterile 1.5 ml tubes. 25 µl of the tested compound were added to the 475 µl of calibrated culture. Low to mid concentrations of chemicals non- toxic for human cells were applied; thus, inhibitory drug concentrations were not determined.

Agents with confirmed and possible *in vitro* activity against the *Acanthamoeba* protozoans were applied.

Povidone Iodine Solutio 10% Teva (PI) (Teva Pharmaceuticals, Poland), a complex of iodine, the bactericidal component with povidone, disinfectant and preoperative antiseptic was dissolved to a final concentration of 0.5% (5 mg/ml).

Chlorhexidine digluconate (CXD), a standard antiseptic belonging to the biguanide family, was used as the reference drug against *Acanthamoeba*. 20% solution (Zakłady Farmaceutyczne Polfa, Łódź, Poland) was dissolved in purified water and a final concentration of 0.02% was applied in these investigations.

Toyocamycin (Toyo), an adenosine analogue selected for this research as an agent with confirmed anti-protozoan activity, (but not for *Acanthamoeba* spp.), synthesized according to Sharma et al. [34] was tested (kindly provided by Prof. Zygmunt Kazimierczuk from the Division of Organic and Food Chemistry, Warsaw University of Life Sciences). Working stock solution in a concentration 1000 μM /ml was prepared by dissolving the compound powder in 9.6% ethanol. The final concentrations of toyocamycin, 25μM/ml and 50μM/ml were obtained by dissolving the working stock in purified water. In addition, purified water and 0.48% ethanol-solvent were added to control cultures containing only amoebae, and subjected to the same procedure used for experimental cultures.

The effect of the compounds on *Acanthamoeba* strains was assessed for each strain following 24h, 48h, 96h, 120h and 144h exposure, and compared with the control culture count considered as 100%. The level of statistical significance was set at $p < 0.05$.

The dynamics of amoebic strains were monitored *in vitro*. A range of amoeba number of two or three counts with the use of Bürker's hemocytometer, calculated for 1 ml of culture medium, was assessed in subsequent days of the exponential growth phase. The percentage of particular stages: trophozoites and cysts, morpho-physiological status of these amoebic forms, and particularly *in vitro* viability of amoebae from both strain populations, were monitored. All assays were repeated twice or thrice; results were analyzed statistically (ANOVA, Student-Newman-Keuls). Values of $p < 0.05$ were considered significant.

RESULTS

Examination of the material acquired from corneal scrapings with the use of a light microscope revealed live trophozoites forming acanthopodia characteristic for *Acanthamoeba* genus, and also double-walled cysts. The environmental *Acanthamoeba castellanii* Neff strain and the corneal strain included in this investigation monitored *in vitro* showed low amoeba number in the early adaptive phase of cultivation that successively increased in the log, exponential growth phase.

Specific identification of the corneal isolate performed using molecular techniques based on genotype associations of the 18S rRNA gene sequence, following the protocol established by Schroeder et al. [33], indicated that the causative agent of severe AK course is the *Acanthamoeba* strain determined as *A. polyphaga* Page-23; ATCC 30871 representing T4 genotype, similar to the genotype of *A. castellanii* Neff maintained and monitored for years in our laboratory [27, 28, 32].

There were statistically significant differences in the log growth phase in the density of the protozoan population at 26 °C: viable amoeba count range of the environmental strain was distinctly higher than the number of the eye isolate, 10–18 x 10^4 and 1–5 x 10^4, respectively ($p < 0.05$). The majority (95%- 98%) were the trophozoite forms.

Effect of chemicals on *Acanthamoeba* environmental and corneal strains. The chemicals investigated showed anti-amoebic effects with various degrees of effectiveness, although the ethyl alcohol concentration had no significant influence on amoebae in all assays. Differences were observed in the population dynamics of *A. castellanii* Neff strain and *A. polyphaga* T4 genotype exposed to povidone iodine or toyocamycin in comparison to the chlorhexidine digluconate which at was taken as the reference.

An amoebostatic activity of all agents tested was revealed but it was not connected with intense amoebic encystation expressed as increased statistically significant cyst frequency; none of the compounds in the concentration applied in this study induced this process. The amoebic cyst percentage was variable throughout the duration of the experiment – around 0.0 -5.2%, because the differences revealed in cultures of particular *Acanthamoeba* strains were insignificant statistically for all agents applied (not presented in detail).

Comparative assessment of the *in vitro* cultivated *Acanthamoeba* strain populations after exposure to chemicals showed changes in morpho-physiological status, number of trophozoites and cysts, as well as in proportion between developmental forms, in comparison to the respective control cultures. The changes were expressed as differences in dynamics and viability of amoebic strains examined after 24h, 48h, 96h, 120h and 144hrs exposure to tested chemicals.

Anti-amoebic effects against *A. polyphaga* T4 genotype were detected after different time exposure to the particular tested agents.

Chlorhexidine digluconate is an antiseptic /disinfectant with a broad spectrum of activity and confirmed effectiveness against *Acanthamoehba* strains. In the beginning, throughout the 4 days after exposure, a variable influence of the chemical on the corneal strain was observed. A clear, statistically significant amoebostatic effect of the agent was revealed just after 120h incubation. CXD is used as a topical drug in AK therapy and for this reason, the results obtained for this chemical were included as the reference, and the effects of povidone iodine and toyocamycin on the amoebae were compared to those revealed for CXD.

There were differences in the dynamics and viability of corneal and environmental amoebic populations examined microscopically after 24, 48, 96, 120 and 144hrs exposure to particular chemicals. Statistically significant changes in the percentage of viable amoebae in comparison to data regarding cultures not exposed to these chemicals appeared from 48h and 96h for Toyo and 96h for PI, compared to 120h for CXD (Tab. 1).

Moreover, microscopic examination of samples of the amoebic cultures revealed that their exposure to the tested chemicals caused changes in the morpho-physiological status of amoebic populations in comparison with that observed in the control cultures. This was expressed not only as a decrease in the general number of amoebae, but also in the sporadic appearance of dividing trophozoites as well as in the frequent occurrence of rounded forms, moving slowly or motionless, without forming acanthopodia.

The clear, statistically significant amoebostatic effect on the eye amoebic strain was revealed in assays with PI. This was reflected as a decrease in the number of live amoebae to 30.8%, observed after 4 days of exposure, in comparison to specimens counted in control cultures that were assumed as 100% ($p < 0.05$). A similar level of the number of live

amoebae (29.9%) was achieved after 5 days of exposure in assays with CXD. More reduced number of the amoebae to 16.7% and 22.2% ($p < 0.05$) that may be considered as anti-amoebic effect of PI was detected in 5 and 6 days exposure to this agent with cultivated amoebae of the corneal isolate to this agent (Tab. 1).

Table 1. *In vitro* sensitivity of *A. polyphaga* T4 corneal strain to tested agents

Live amoebae after exposure to the agents* by the following hours:						
Agent	*Concen-tration*	*24h*	*48h*	*96h*	*120h*	*144h*
Povidone iodine	0.5%	100%	89.9%	30.8%	16.7%	22.2%
Chlorhexidine. digluconate	0.02%	74.%	75%	68.2%	29.9%	28.8%
Toyocamycin	25 µM/ml	117.6%	84.4%	47.2%	53.4%	37.4%
	50 µM/ml	116.2%	74.3%	24.8%	44.5%	24.8%

*) Percentage of viable amoebae in comparison to specimens count in control cultures assumed as 100%.
Level of statistical significance set at $p < 0.05$. Statistically significant differences in relation to data regarding cultures not exposed to chemicals are underlined.

Anti- amoebic activity on the corneal *Acanthamoeba* strain was also revealed in tests with toyocamycin. The statistically significant reduction of the pathogenic amoeba population density was more intense in cultures exposed to Toyo in the concentration of 50 µM/ml than that of 25 µM/ml. Simultaneously, the decrease in the number of live amoebae was observed earlier after exposure to Toyo for 48h and 96h, in comparison to the 120h observed in cultures exposed to CXD. However, a variable intensity of amoebostatic impact of Toyo on the amoebic populations was revealed during subsequent monitoring days (Tab. 1). Contrary to this observation, anti-amoebic activity of PI resulted in a consistent downward trend in corneal *Acanthamoeba* strain population density.

Protists of the *A. castellanii* Neff strain indicated more variable sensitivity to the tested agents than that of the corneal *Acanthamoeba* strain (Tab. 2); this was particularly significant in assays with PI and Toyo. The amoebostatic effect of the iodophore was expressed in a significantly reduced percentage of viable amoebae after120h exposure, up to 14% in comparison to specimens counts in control cultures assumed as 100%. This effect of PI was more intense than the impact of CXD – 30.8%, ($p < 0.05$). In contrast, the amoebae of the strain were clearly more resistant to Toyo than to CXD, e.g. the percentage of live amoebae at 96h exposure to this chemical decreased to the level 62.5%, in comparison to 18.2%, respectively ($p < 0.05$).

Table 2. *In vitro* effect of tested agents on *Acanthamoeba castellanii* Neff strain

Live amoebae after exposure to the agents* by the following hours:						
Agent	Concen-tration	24h	48h	96h	120h	144h
Povidone iodine	0.5%	63.7%	33%	18.2%	14%	14.3%
Chlorhexidine. digluconate	0,02%	84%	67.8%	39.6%	30.8%	31%
Toyocamycin	25 µM	97.7%	77.83%	73.50%	89.7%	96.24%
	50 µM	83.2%	70.8%	62.5%	87.5%	74%

* Percentage of viable amoebae in comparison to specimens count in control cultures assumed as 100%.
Level of statistical significance set at $p < 0.05$. Statistically significant differences in relation to data regarding cultures not exposed to chemicals are underlined.

In vitro sensitivity of *A. polyphaga* T4 corneal strain and *A. castellanii* Neff environmental strain to the tested agents is presented in Tables 1 and 2.

DISCUSSION

Acanthamoeba keratitis is an emerging, sight-threatening eye disease caused by the facultative parasites of the genus *Acanthamoeba*, which are ubiquitous in human environments. Without adequate therapy, the amoebic infection that causes a significant deterioration of visual acuity, may lead to blindness.

The knowledge and awareness of vision-threatening *Acanthamoebic* keratitis as a serious corneal disease are still insufficient, and during the last few decades the cases of AK have been constantly increasing worldwide [6–9, 13, 15–17, 23–25, 30].

The leading risk factor for AK is contact lens wear; a corneal epithelial injury and exposure of the eye to water containing cysts and trophozoites of amphizoic amoebae, apart from contact lens wear, are other factors predisposing to AK [8–10, 15–17, 30].

Diagnostic errors that delay appropriate treatment may result in a prolonged, severe course of AK. It is emphasized in the literature and confirmed by our experience [13–17, 28, 30] that the eye disease caused by the *Acanthamoeba* protozoans is often misdiagnosed due to unspecific clinical symptoms, and then unsuccessfully treated as a viral infection with *Herpes simplex,* bacterial keratitis caused by *Pseudomonas aeruginosa* or fungal infection caused by *Fusarium* spp. or *Candida* spp. Moreover, the mixed keratitis caused by amoebae and concomitant bacterial, viral, or fungal infections may also occur. Non-invasive methods are recommended for preliminary rapid diagnostics, e.g. using the slit lamp for disclosure of an active epithelial inflammation and a visualization of hyper-reflective objects, e.g. cysts of *Acanthamoeba* by *in vivo* confocal microscope [35, 36, 37].

Laboratory microbiology and parasitology examinations of corneal scraping samples by light microscopy are applied for the detection and determination of *Acanthamoeba* species, and the culture methods are considered the gold standard for the verification/confirmation of the diagnosis [15, 16, 17, 28, 30, 35–42]. For years, the diagnostics were based on the structure of the amoebic developmental forms. Three morphological groups of species were established within the *Acanthamoeba* genus based on morphological criteria, mainly cyst size and the number of arm-like structures within a single cyst [14, 15, 16, 17, 30]. However, pathogenic *Acanthamoeba* may belong to species similar morphologically, i.e. variation in the cyst morphology occur in *A. mauritaniensis*, *A. castellanii, A. polyphaga*, and *A. hatchetii*, and additional isolates may be revealed.

Recently, with the development of molecular techniques, the classification of clinical and environmental strains is based on associations of 18S rRNA gene sequences, PCR products analysis and sequencing. Obtained sequences are compared with data available in GenBank to determine genotypes of the individual isolates [14–17, 26, 27, 29, 33]; to-date, 18/19 genotypes have been determined [15, 17, 30].

Apart from misdiagnosis, many factors complicate effective pharmacotherapy in *Acanthamoeba* keratitis. Different

viability of amoebic strains, their various susceptibility/ resistance to chemicals, unequal impact of drugs on trophozoites and cysts, various responses of amoebae to formerly effective pharmaceuticals, and the toxicity of chemicals to human corneal cells in effective concentrations result in treatment difficulties. For this reason, the search for substances with possible activity against *Acanthamoeba* strains is still necessary.

Previous studies by the authors of the current study have shown that cultivation and *in vitro* monitoring of population dynamics of amoebic strains isolated from the infected corneas may be useful tools for assessment of the viability and changes in morpho-physiological status of the etiological agent of AK, and for relating the data to a prognosis of pharmaceutical efficacy in the disease [28, 30]. In the presented study, the amoebae of corneal *A. polyphaga* T4 genotype derived from an eye with a clinically severe course of AK, complicated diagnostically and therapeutically, was assessed. A strong *in vitro* viability of the amoebae was expressed in their long surviving time linked with the ability of trophozoites to multiply in the axenical culture medium. Moreover, during *in vitro* monitoring of amoebic population dynamics, significant differences were revealed in resistance/ sensitivity of the corneal strain to the agents tested.

At present, no single chemical has been described as an effective treatment against AK. The selection of drugs to be used and the duration of AK therapy may depend on the

diagnosis time-point, drug resistance of the isolated *Acanthamoeba* strain, severity of infection related to the amoebic strain viability, and the prevalence of other infectious microorganisms. It has been found that pathogenic isolates may differ in susceptibility to chemical agents/drugs; it is also known that isolates within the same genotype may differ in susceptibility to chemicals [15, 17, 26, 30, 40]..

The treatment of AK has not yet been fully established. Combination drug therapy is used more or less successfully and mainly involves the topical application of chlorhexidine digluconate, propamidine isethionate (Brolene) and polyhexamethylene biguanide (PHMB), with an addition of antibiotics, such as neomycin or chloramphenicol, to avoid possible bacterial infections [15, 22, 43]. Results of applied therapy are often disappointing due to the resistance of amoebae to disinfectants and drugs. It is emphasized that the extremely high resistance of double-walled *Acanthamoeba* cysts to chemicals, anti-microbial and anti-parasitic drugs, is considered one of the key contributors of treatment failure [15, 22, 31, 38, 43].

Many chemicals have been tested *in vitro* for their anti-amoebic activity and different

Acanthamoeba strains/ isolates of various pathogenicity assessed for their susceptibility /resistance to disinfectants, antiseptics and drugs, including the presented study with nanoparticles as novel therapeutic agents [15, 17, 39–46].

Povidone iodine solution is an agent with broad antibacterial and antiviral activity applied in wound care treatment and preoperative antisepsis. Gatti at al. [40] in their study undertaken in terms of *in vitro* effectiveness of PI against *Acanthamoeba* strains, including *A. polyphaga,* showed some sensitivity of particular amoeba strains to the agent in a solution of 0.5 – 2.5%, depending on the disinfectant concentrations and type of medium applied for dilutions. The study showed that the PI system is an effective disinfection method for contact lenses. Currently, it is recommended as a contact lens care disinfectant, and also for use as eye drops in some ophthalmic diseases [40–42].

In the current study, a clear, statistically significant amoebostatic effect of povidone iodine has been revealed on both *Acanthamoeba* strains.

Chlorhexidine digluconate, a cationic antiseptic utilized in prevention and surgeries is currently used in the AK therapy and applied in a concentration of 0.02%. It inhibits membrane function and has been shown previously to have a good *in vitro* anti-*Acanthamoeba* activity [15, 17, 40, 43]. However, in spite of the overall amoebicidal *in vitro* effects, a significant increase in the amoeba cysts level up to 11.6% was also observed at higher concentrations of the agent, as well as the AK chemotherapeutic resistance or non-responsiveness to CXD [17, 30, 42]. CXD showed moderate amoebostatic effect on the eye amoebic strain.

In the presented study, 0.02% chlorhexidine activity was also tested against two *Acanthamoeba* strains. The obtained results were taken as a reference, and the effects of povidone iodine and toyocamycin on the amoebae were compared to those obtained for chlorhexidine digluconate.

Toyocamycin is the adenosine analogue derived from *Streptomyces toyocaensis;* it blocks the RNA synthesis and ribosome function. A criterion selection of the agent for the current study was its confirmed anti-protozoan activity against *Trichomonas* sp. and *Toxoplasma* sp. [43].

To the best knowledge of the authors of the presented study – apart from their preliminary research [44] – this is the first such study undertaken in Poland with diagnosed, pathogenic *Acanthamoeba* strains leading AK in terms of their *in vitro* susceptibility to the anti-protozoan agent toyocamycin.

CONCLUSION

The presented study examined and assessed the anti-amoebic *in vitro* effects of selected chemicals on the etiological agent of sight-threatening eye disease, *Acanthamoebic* keratitis, and the influence of the agents on non-pathogenic environmental *A.castellanii* Neff T4 genotype. It revealed time-dependent amoebostatic *in vitro* influence of all agents on the *Acanthamoeba* strains investigated with various degrees of effectiveness. Comparative assessment of the *in vitro* cultivated *Acanthamoeba* strains after exposure to the chemicals showed changes in the morpho-physiological status of the populations and number of trophozoites and cysts, as well as the proportion between developmental forms in comparison to the respective control cultures.

The results of the study also revealed that povidone iodine has a better anti-amoebic activity on trophozoites of *Acanthamoeba* spp. than CXD. Toyocamycin, chemicals with confirmed anti-protozoan effectiveness, was tested in terms of their potential *in vitro* activity against the *Acanthamoeba* strains. The agent also indicated that an anti- amoebic effect was reached faster than in samples with CXD; however, this impact was weaker than the influence of IP.

In conclusion, it is noteworthy that none of the tested chemicals in applied concentration induced encystations; simultaneously, no cysticidal effects were caused by these compounds. However, it should be taken into consideration that prolonged *in vivo* treatment may induce encystations, an undesirable process predisposing to disease recurrence. For this reason, further *in vitro* investigations on various

species/strains/isolates of *Acanthamoeba*, etiological agents of the sight-threatening AK are still necessary. In particular, complementary research should be conducted with different chemicals, modified agent concentrations and application methods in terms of their potential *in vitro* amoebicidal / cysticidal efficacy, to determine the next possibility for the effective treatment of AK.

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