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In vitro validation of the amoebicidal activity of commercial eye drops as second activity

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ABSTRACT

The validation of anti-*Acanthamoeba* activity of commercial eye drops has gained a great interest recently and a growing number of commercials eye drop were evaluated for their aptitude to inhibit *Acanthamoeba* as a second pharmacological effect. In the present study, three different eye drops, commercializing in Spain, including TobraDex, Cusimolol and Colircusi antiedema have been tested *in vitro* against trophozoites and cysts stage of the facultative pathogen *Acanthamoeba*. The alamarBlue™ method was used to evaluate both trophocidal and cysticidal properties. The most active eye drops were tested for their impact on some programmed cell death features. We found out that the cells inhibition was strain and eye drop dependent, and 5% eye drop was able to eliminate both trophozoite and cyst stage of *Acanthamoeba* spp. A treatment of 24 h with 5% of TobraDex or Cusimolol was able to show DNA condensation, collapse in the mitochondrial membrane potential and reduction of the ATP level production in *Acanthamoeba*. We could assume that the present eye drops could induce programed cell death like process in *Acanthamoeba* via mitochondrial pathway.

1. Introduction

Several common eye disorder or diseases can happen frequently. They can be of minor gravity such as eyestrain and dry eyes syndrome. Other can be more serious and require long term treatment, surgery or can even be uncurable.

Among these diseases, keratitis caused by the genus *Acanthamoeba* spp also known as *Acanthamoeba* Keratitis (AK). This parasitic eye infection is described since 1973. It manifests on corneal lesion caused by the presence of these protozoa. These lesions are invasive and can induce sever sequel reaching even blindness (Lorenzo-Morales, J. et al., 2013).

Keratitis due to *Acanthamoeba* spp are common in contact lenses wearers and this incidence in this specific population is mainly due to the hygiene associated to their use. Even though, cases have been detected in people not using contact lenses, the use of this ocular prosthetic device have been proved to be involved in the increase of the incidence of this sight-threatening diseases (Lorenzo-Morales, J. et al., 2015).

The fact that makes keratitis induced by *Acanthamoeba* spp difficult to treat is the capacity that have the parasite to transform to a resistant form known as cyst. And the survival after treatment of even one cyst in

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the cornea can produce a second infection (Lakhundi et al., 2017). This characteristic involves the need of monitoring of treatment and checks even after recovery. And this also urge the need to find an efficient treatment that can affect both forms of *Acanthamoeba* spp, being nontoxic for the host and induce a controlled cell death on parasitic cells.

The pharmaceutical drug market is full of treatment oriented to eye disorder or diseases. They can be under different dosage forms. Semi-solid ophthalmic drug form like ophthalmic *in situ* gel or eye ointment. Eye treatment can also be administrated via solid form using ocular insert and contact lenses coated with drugs (Mazet et al., 2020). Eye treatment can also be under liquid form which is the most conventional one and among this group eye drops account for 90% of the marketed ophthalmic formulations (Patel et al., 2013). Eye drops are a water and oil that can be in solution, emulsion or suspension of active compounds. They are sterile and isotonic. If they are marketed in multiuse packaging, a preservative is added to the formulation of the product (Baranowski et al., 2014).

Eye drops solution are largely used due to their safety, immediate activity and the administration mode that is considered as non-invasive for patient and among the large offer available in pharmaceutical markets, we selected three products to evaluate their effects against different species of genus *Acanthamoeba.*

TobraDex is an eye drop suspension manufactured by Alcon, a global leader in eye care. Each mL of TobraDex solution contain 3 mg of tobramycin and 1 mg of dexamethasone associated to 0.1 mg of benzalkonium chloride used as excipient. it is used to prevent and treat inflammation and to prevent infections associated with cataract surgery in adults and children aged 2 years and older. The usual posology is a drop each 4–6 h during the day for at least 14 days and not more than 24 days for adults.

The second selected eyed drop is Cusimolol. The formulation of the product is commercialized in Spain by Novartis Farmaceutica as ophthalmic solution. The active compound contained in the solution is timolol under the form of timolol maleate at a concentration 5 mg/mL the solution contains also benzalkonium chloride at 0.01% as preservative. Cusimolol is used as anti-glaucoma drug and is classified among non-selective beta-blocker and is prescribed to reduce intraocular pressure. The recommended posology is two drops per day.

Colircusi antiedema is the third eye drop to be tested against *Acanthamoeba* spp. It is also marketed in Spain by Novartis Farmaceutica. The active substance contained in the eye drop is sodium chloride at a concentration of 50 mg/mL. Sodium chloride being under the form of hypertonic solution, when it gets in contact with corneal epithelium, induce water extraction from the cornea which maintain it without inflammation. The use of this eye drop is recommended in case of corneal edema which manifest by the accumulation of liquid in the cornea. The usual posology of Colircusi antiedema is 1–2 drops in the eye to treat each 3–4 h.

In the current work, we evaluated the amoebicidal activity of the three previously cited eyedrops against different species of *Acanthamoeba* which are *A. castellanii* Neff, *A. polyphaga, A. griffini* and *A. quina.* The *in vitro* evaluation of the activity of these three marketed products was performed against both trophozoite and cyst stage. We also detected several biological and molecular events in order to elucidate the cell death mechanism that these products will induce in the parasite such as chromatin condensation, membrane permeabilization or ATP production.

2. Material and methods

2.1. Chemical

In the present study the three commercial eye drops TobraDex, Cusimolol and Colircusi antiedema (Alcon a Novartis company, Barcelona, Spain), were selected to evaluate its anti-*Acanthamoeba in vitro* activity.

2.2. Acanthamoeba strains

The trophocidal activity of the three eye drops was made against four strains of *Acanthamoeba: Acanthamoeba castellanii* Neff, genotype T4 (ATCC 30010) type strain from the American Type Culture Collection; *Acanthamoeba griffini*, genotype T3 obtained in previous studies (González-Robles et al., 2014); *Acanthamoeba polyphaga*, genotype T4 (ATCC 30461) and *Acanthamoeba quina,* genotype T4 (ATCC 50241) (Figure SM1). Those strains were grown axenically in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose) containing 40 μg gentamicin mL-1 (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

2.3. In vitro effect against the trophozoite stage of Acanthamoeba

The anti-*Acanthamoeba* activity of the all the eye drops solution were determined by the alamarBlue™ assay (life technology; Madrid; Spain) as previously described (Martín-Navarro et al., 2008; Sifaoui et al., 2017; Sifaoui et al., 2018). Briefly, *Acanthamoeba* strains were seeded in triplicate on a 96-well microtiter plate with 50 μL from a stock solution of 5×10^4 cells/mL. Amoebae were allowed to adhere for 15 min and 50 μL of serial dilution series of the drug was added. Finally, the alamarBlue™ Cell Viability Reagent was added into each well at an amount equal to 10% of the medium volume. The plates were then incubated at 28 °C with a slight agitation and the emitted fluorescence was measured after 4 days of incubation with an EnSpire microplate reader (PerkinElmer, Massachusetts, USA) at 570/585 nm.

2.4. In vitro effect against the cyst stage of Acanthamoeba spp

The cysticidal activity was determined by the alamarBlue™ assay after 7 days of incubation and confirmed visually by inverted microscopy (Leica DM IL; Wetzlar, Germany). Mature Cysts of all the strains were prepared as described by Lorenzo-Morales et al. (2008) (Lorenzo-Morales et al., 2008). Briefly, trophozoite were transferred from PYG medium based cultures (trophozoite medium) to Neff's encystment medium (NEM; 0.1 M KCl, 8 mM $MgSO_4{\cdot}7H_2O$, 0.4 mM $CaCl_2{\cdot}2H_2O$, 1 mM $NaHCO_3$, 20 mM ammediol [2-amino-2-methyl-1,3-propanediol; Sigma Aldrich Chemistry Ltd., Madrid, Spain], pH 8.8, at 25 °C) and were cultured in this medium with gently shaking for a week in order to obtain mature cysts. After that, mature cysts were harvested and washed twice using PYG medium.

A serial dilution of the eye drops was made in PYG. The *in vitro* susceptibility assay was performed in sterile 96-well microtiter plates (Corning life science; MA; US). To these wells the drug concentration to be tested and 5×10^4 mature cysts of *Acanthamoeba*/mL were added. The final volume was 100 μL in each well. The plates were examined with inverted microscopy, after 7 days of incubation, the plates were centrifuged at 3000 rpm and the medium was replaced with a new PYG. Finally, 10 μL of the alamarBlue™ Cell Viability Reagent was placed into each well, and the plates were then incubated at 28 °C with slight agitation and the emitted fluorescence was examined after 7 days of incubation with an EnSpire microplate reader (PerkinElmer, Massachusetts, USA) at 570/585 nm.

2.5. Measurement of ATP

ATP level produced during the time was measured using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madrid, Spain). The effect of the present eye drop on the intracellular ATP production was evaluated by incubating (10^5) of cells/mL in 96 MicroWell white polystyrene plate (Thermo Scientific Nunc, Madrid, Spain) with the 10% of the eye drops (Sifaoui et al., 2018). After 24 h of incubation, 100 μL of CellTiter-Glo® Reagent were added to each well. The plate was mixed

for 2 min on an orbital shaker to induce cell lysis. Plate were incubated at room temperatures for 10 min and the luminescence was measured with the EnSpire microplate reader.

2.6. Double-stain assay for programmed cell death determination

A double-stain apoptosis detection kit Chromatin Condensation/ Dead Cell Apoptosis Kit with Hoechst 33,342 and PI (Invitrogen, Madrid, Spain) and an EVOS FL Cell Imaging System AMF4300 (Life Technologies, Madrid, Spain) were used. The experiment was carried out by following the manufacturer's recommendations, and 10^5 cells/well were incubated for 24 h with the 10% of the present eye drop. The double-staining pattern allows the identification of three groups in a cellular population: live cells shows only a low level of fluorescence, cells undergoing PCD shows a higher level of blue fluorescence (as chromatin condenses), and dead cells shows low-blue and high-red fluorescence (as the Propidium Iodide stain enters the nucleus) (Cartuche et al., 2019).

2.7. Intracellular ROS production using CellROX® Deep Red staining

The generation of intracellular ROS was detected using the CellROX® Deep Red fluorescent probe (Invitrogen, Madrid, Spain). The cells were treated with the 10% of the eye drop for 24 h and exposed to CellROX® Deep Red (5 μM, 30 min) at 26 °C in the dark. Cells were observed with an EVOS FL Cell Imaging System AMF4300 (Life Technologies, CA, USA) (Cartuche et al., 2019).

2.8. Analysis of mitochondrial membrane potential

The collapse of an electrochemical gradient across the mitochondrial membrane during the time was detected with the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman, Madrid, Spain). After being treated with 10% of eye drop solutions for 24 h, the cells were centrifuged (1500 r.p.m. × 10 min) and resuspended in JC-1 buffer. After that, 50 μL of each treated culture was incubated with 5 μL of JC-1 at 26 °C for 30 min. Images were taken on an EVOS FL Cell Imaging System AMF4300 (Life Technologies, CA, USA). The staining pattern allows the identification of two groups in a cellular population: live cells shows only red fluorescence; cells with low mitochondrial potential, (undergoing PCD) shows a higher level of green and red fluorescence (Cartuche et al., 2019).

2.9. Plasma membrane permeability

The SYTOX® Green (Molecular Probes, Invitrogen, Madrid, Spain) assay was performed to detect membrane permeability damages in treated cells. Briefly, 10^5 trophozoite were incubated for 24 h with the 10% of the drug solution. Subsequently, the SYTOX® Green dye was added at a final concentration of 1 μM for 15 min in the dark. Cells were observed in an EVOS FL Cell Imaging System AMF4300 (Life Technologies, CA, USA) (Cartuche et al., 2019).

2.10. Statistical analysis

All data were recorded, edited and entered using GraphPad Prism version 8.0 (GraphPad Software; CA; USA). The data.are expressed as the mean ± standard deviation of at least three independent experiments. To highlight the effect of the type strain and type of eye drops used on *Acanthamoeba* spp viability. A statistical analysis was conducted using two-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

3. Results

- *In vitro activity against Acanthamoeba* spp

The evaluation of *in vitro* activity of the three selected eye drops have been evaluated against different species belonging to the genus *Acanthamoeba (A. castellanii* Neff, *A. polyphaga, A. griffini* and *A. quina)*. The IC_{50} was determined for both trophozoite and cyst stage and results are summarized in Table 1. Two-way ANOVA analysis was applied to study the effect of type strain and eye drop on the trophocidal and cysticidal activities, both factors and their interaction have a significant effect on the inhibition of trophozoite and cyst with $p < 0.001$. Statistical analysis using one-way ANOVA test allowed us to evaluate significant effect of TobraDex, Cusimolol and Colircusi toward each species of *Acanthamoeba* and each stage.

The obtained values of IC_{50} allowed us to conclude that *A. castellanii* Neff seems to be the most sensitive to the three eyedrops tested with values of 1,56%, 2,80% and 2,37% for TobraDex, Cusimolol and Colircusi antiedema respectively. The effect of the type of eye drop tested was statistically significant in all the strains. According to the data presented in Table 1, it is visible that the commercialized eye drop TobraDex is the most active against *A. castellanii* Neff, *A. polyphaga, A. griffini* and *A. quina* with IC_{50} ranging from 1.56% to 3.16%.

- ***In vitro activity against cyst stage***

Results obtained for the evaluation *In vitro* of the activity of the three commercialized eyedrops tested against cyst stage of different species of *Acanthamoeba* exhibited variable capacities and a significative effect of the product tested As presented in Table 1, it is clear that TobraDex is the most active against cysts of *A. castellanii* Neff and *A. polyphaga* with IC_{50} values of 1.51% and 2.68% respectively with a significative difference with Cusimolol and Colircusi antiedema. This tendency is different for the strains *A. griffini* and *A. quina* for which obtained values of IC_{50} indicate that Cusimolol is the most potent against cyst stage of the two strains even though the difference for *A. quina* is not statistically significative between Cusimolol and TobraDex.

- **Elucidation of cell death mechanisms**

3.1. Measurement of ATP

Production of ATP have been quantified in control cells untreated and cells incubated for 24 h with the 10% of different eyedrops tested and the effect was estimated as a decrease of the amount of ATP produced compared to untreated control cells. The results obtained are represented in Fig. 1. Reduction on ATP production have been induced by the three products but with different intensities. A similar tendency has been observed in the four *Acanthamoeba* strains (*A. castellanii* Neff, *A. griffini, A. polyphaga* and *A. quina)*. Statistical analysis of obtained results led to conclude that a significative difference exists between the three eyedrops tested against all the strains. As represented in Fig. 1, the eyedrop Cusimolol in the product that affected the most *Acanthamoeba* spp cells with values under 2% for *A. griffini* (a loss of 98% of production) and 15.7% for *A. quina.* according the obtained results, this last seems to be the less affected by all products tested. In the other hand, the commercialized eyedrop under the name Colircusi is the one that affected less the production of ATP with a reduction not exceeding 50%, considering this, the next assays have been performed only with the products Cusimolol and TobraDex.

A double staining has been performed in order to detect on treated cells chromatin condensation (blue fluorescence) and cell death (red fluorescence). Obtained results are represented in Fig. 2 were control cells of different *Acanthamoeba* strains are compared to cells incubated 24 h with 10% of TobraDex and Cusimolol. As can be observed in the images, control cells don't exhibit any blue fluorescence at the opposite of cells treated with the two tested eyedrops. The obtained results suggest a stronger chromatin condensation and more dead cells for all strains under the effect of Cusimolol with a higher proportion of

Table 1
Amoebicidal activities of eye drops on *Acanthamoeba* spp. (IC_{50} are expressed in % v:v).

	Acanthamoeba castellanii Neff		*Acanthamoeba polyphaga*		*Acanthamoeba griffini*		*Acanthamoeba quina*	
	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst
Tobdradex	1.56 ± 0.36^{a}	1.51 ± 0.00^{A}	1.73 ± 0.09^{a}	2.68 ± 0.03^{A}	3.16 ± 0.48^{a}	5.37 ± 0.10^{B}	1.68 ± 0.16^{a}	4.48 ± 0.09^{A}
Cusimolol	2.80 ± 0.13^{b}	2.52 ± 0.34^{B}	3.74 ± 0.20^{b}	4.43 ± 0.01^{B}	3.95 ± 0.16^{a}	4.18 ± 0.13^{A}	2.96 ± 0.12^{b}	3.84 ± 0.64^{A}
Colicursi	2.37 ± 0.04^{ab}	2.67 ± 0.19^{B}	4.58 ± 0.05^{c}	$>10^{C}$	4.90 ± 0.33^{b}	$>10^{C}$	1.65 ± 0.56^{ab}	$>10^{B}$

letters a-c; reflect that means of trophocidal activity within the same strains with different letters are significantly different ($p < 0.05$).
letters A-C; reflect that means of cysticidal activity within the same strains with different letters are significantly different ($p < 0.05$).
- *In vitro* activity against trophozoites stage

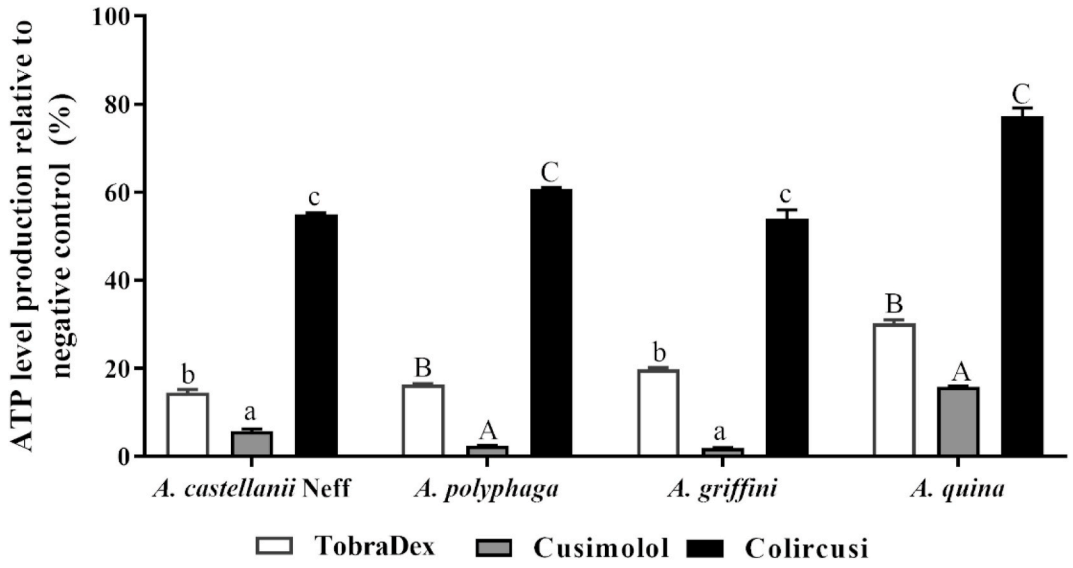

Fig. 1. The effect of eye drops on ATP production of *Acanthamoeba* spp, using CellTiter-Glo® luminescent cell viability assay during incubation time. Results are representing in percentage relative to the negative control. Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3); and letters a-c and A-C reflect that means within strains with different letters are significantly different ($p < 0.05$). Double-stain assay for programmed cell death determination.

fluorescent cells.

3.2. Analysis of mitochondrial membrane potential

The use of JC-1 Mitochondrial Membrane Potential Assay Kit help us to detect eventual collapse of gradient induced by the exposition of cells to two commercialized eyedrops tested and compared to untreated control cells.

A healthy cell would exhibit a red fluorescence meanwhile a collapse depending on its degree will induce a mix between green and red fluorescence. Obtained results are regrouped in Fig. 3 were it is clear that control cells present a red fluorescence and cells treated with the eyedrops exhibit green fluorescence. A difference can be noticed regarding the effect of Cusimolol on *A. castellanii* Neff and *A. quina*. The fluorescence observed is a mix between red and green (orange to yellow color) which is indicator of low mitochondrial potential.

3.4. Intracellular ROS production using CellROX® Deep Red staining

The detection of accumulation of Reactive Oxygen Species at a cellular level is performed using cell imaging after staining untreated control cells and incubated cell with products to test. The red fluorescence detected in obtained images indication production of intracellular ROS. The results obtained and summarized in Fig. 5 clearly indicate that the exposition of different *Acanthamoeba* strains to TobraDex and Cusimolol induced the production of ROS even though the images suggest a stronger effect with the commercialized eye drop Cusimolol.

3.3. Plasma membrane permeability

Membrane permeability being an important indicator of cell health and cell death process, Sytox green assay was used to detect damages induced by the incubation of cell with 10% of TobraDex and Cusimolol. Images obtained are regrouped in Fig. 4.

As can be observed, control cells don't emit any green fluorescence which indicating a perfect membrane integrity. In the opposite, cell imaging illustrates a green fluorescence on the four *Acanthamoeba* strains after treatment for 24 h with both TobraDex and Cusimolol. According to the results obtained, the effect of this last product seems to be more important inducing membrane damages in larger proportion of cells than TobraDex.

4. Discussion

Due to the negative impact of vision loss on quality life eye health has attracted a considerable amount of worldwide attention (Prozesky et al., 2007; Ravilla and Ramasamy 2012). This effect has been associated to most common eye Disorders and Diseases including Refractive Errors, Age-Related Macular Degeneration, Cataract, Diabetic Retinopathy, Glaucoma, Amblyopia and Strabismus among others (World Health Organization 2013; Scott et al., 2016). The increase in incidence and prevalence of chronic eye diseases has inspired the research community to further develop ophthalmic treatments (Research and Markets 2020). Among those diseases, the incidence of *Acanthamoeba* keratitis keeps increasing year after year, mostly in contact lens wearers, although cases have also been reported in non-contact lens wearers (Lorenzo-Morales,

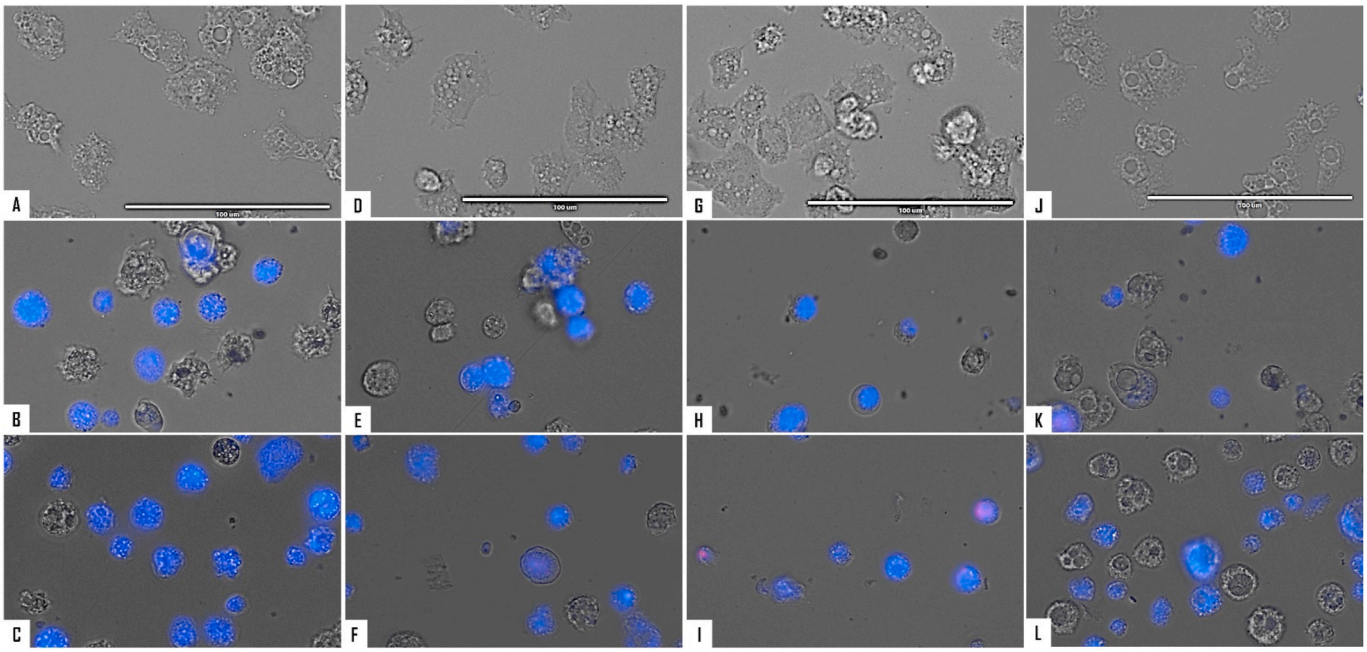

Fig. 2. The effect of 10% of Tobradex (**B, E, H, K**) and 10% of Cusimolol (**C, F, I, L**) on chromatin condensation of *A. castellanii* Neff (**B, C**)*; A. polyphaga* (**E, F**), *A. griffini* (*H, I*) and *A. quina* (**K, L**) trophozoites. The intensity of the Hoechst stain is different in control cells (**A, D, G, J**), cells were unstained while in treated trophozoites cells emits bright blue fluorescence. Red fluorescence corresponds to the propidium iodide stain. Images (40X) are showing chromatin condensation (blue) in *Acanthamoeba* treated cells. Images are representative of the cell population observed in the performed experiments. Images were obtained using an EVOS FL Cell Imaging System AMF4300. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

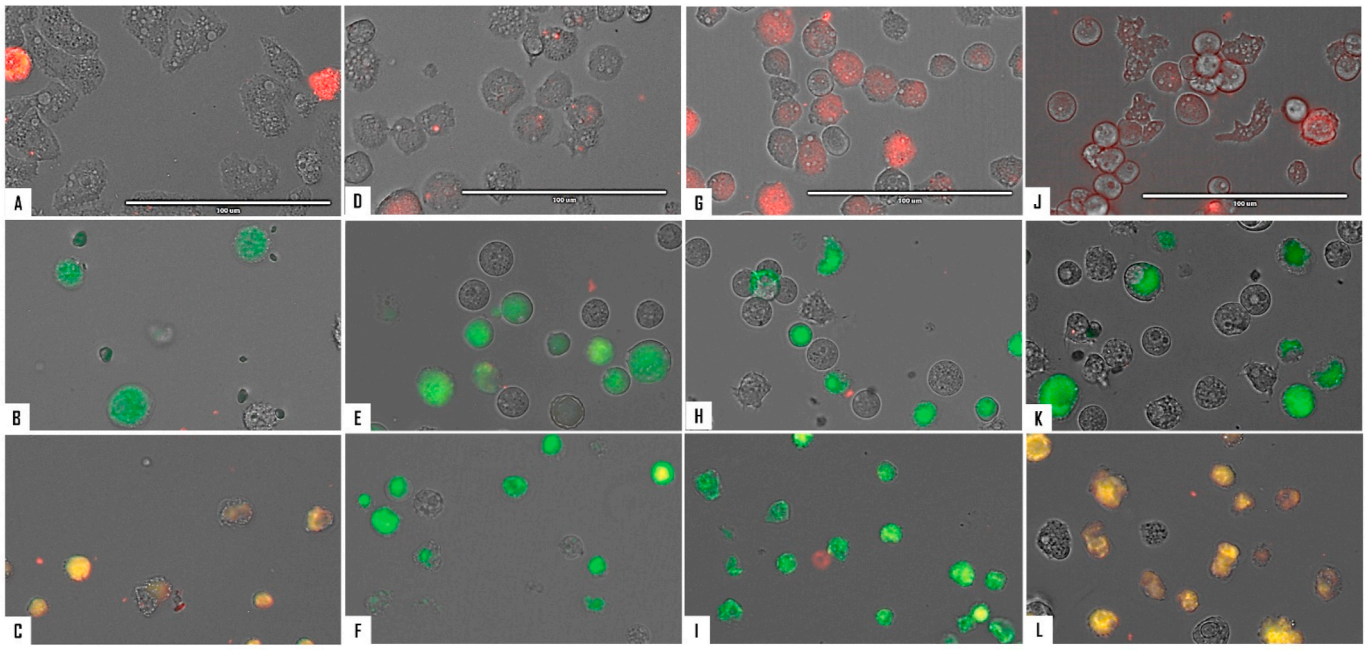

Fig. 3. Images (40X) obtained from EVOS FL Cell Imaging System showing the effect of 10% of Tobradex (**B, E, H, K**) and 10% of Cusimolol (**C, F, I, L**) on the mitochondrial membrane potential of *A. castellanii* Neff (**B, C**)*; A. polyphaga* (**E, F**), *A. griffini* (*H, I*) and *A. quina* (**K, L**) trophozoites compared to the control (**A,D, G, J**) after 24 h of incubation. JC-1 dye accumulates in the mitochondria of healthy cells as aggregates (red fluorescence) (Negative control); in cells treated with the eye drops and due to collapse of mitochondrial potential, the JC-1 dye remained in the cytoplasm in its monomeric form and emit a green fluorescence. Cell emitting both fluorescence will figure in yellow. Images are representative of the cell population observed in the performed experiments. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

J. et al., 2015). The actual treatment protocol is based on the administration several time a day of 0.02% biguanides and 0.1% diamidines (Elsheikha et al., 2020), 0.02%–0.04% chlorhexidine (Dart et al., 2009). Yet, several genotypes have developed resistance to those drugs, or that the efficacy of the drug differs among different genotype and strains. Padzik et al. (2018) (Padzik et al., 2018) have reported that *A. polyphaga*

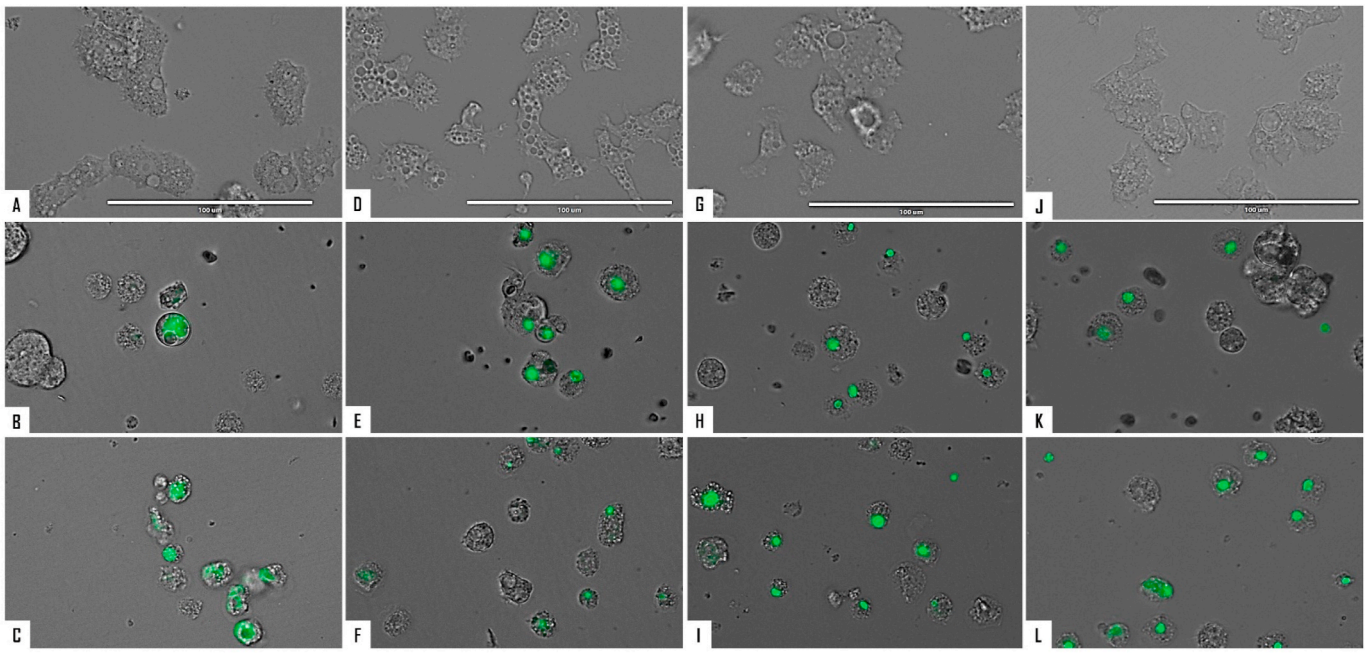

Fig. 4. Images (40X) obtained from EVOS FL Cell Imaging System showing the effect of 10% of Tobradex (**B, E, H, K**) and 10% of Cusimolol (**C, F, I, L**) on the membrane permeability of *A. castellanii* Neff (**B, C**)*; A. polyphaga* (**E, F**), *A. griffini* (**H, I**) and *A. quina* (**K, L**) trophozoites compared to the control (**A,D, G, J**) using Sytox Green dye after 24h of incubation. Sytox Green is a high-affinity nucleic acid stain that penetrates cells with damaged plasma membranes and enhances its fluorescence by more than 500-fold upon nucleic acid binding. Images are representative of the cell population observed in the performed experiments. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

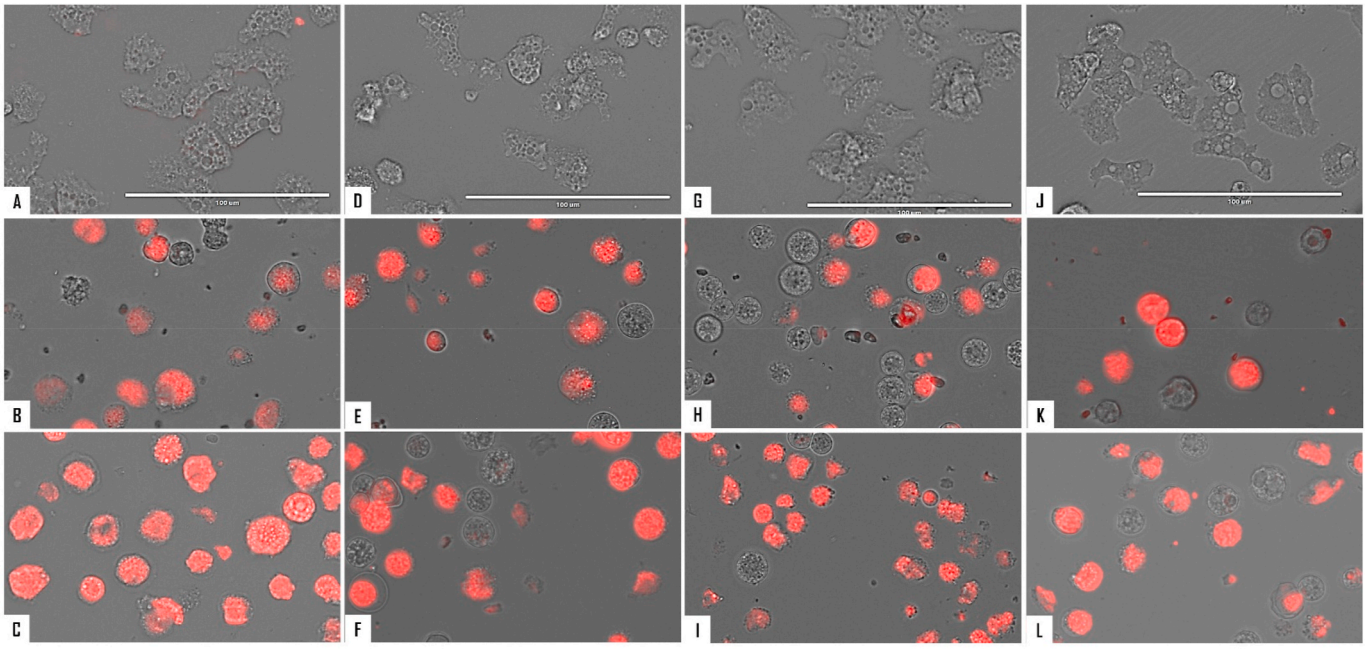

Fig. 5. Images (40X) obtained from EVOS FL Cell Imaging System showing the effect of 10% of Tobradex (**B, E, H, K**) and 10% of Cusimolol (**C, F, I, L**) on the production of Reactive Oxgen species production in *A. castellanii* Neff (**B, C**)*; A. polyphaga* (**E, F**), *A. griffini* (**H, I**) and *A. quina* (**K, L**) trophozoites compared to the control (**A,D, G, J**) after 24 h of incubation. CellROX™ Deep Red Reagent is a cell-permeant dye: non fluorescent in its reducing form and upon oxidation by ROS it emits a bright red fluorescence. Images are representative of the cell population observed in the performed experiments. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was more resistant to chlorhexidine digluconate solution (0.02%) than the environmental *Acanthamoeba* Neff strain. Ferrari et al. (2011) reported a clinical case of AK with *Acanthamoeba* drug resistance towards both polyhexamethylene biguanide (PHMB)-hexamidine and chlorhexidine-hexamidine treatment. However, they notice a combination of both PHMB and chlorhexidine seems to inhibit the parasite in question (Ferrari et al., 2011).

In the present study, the trophocidal and cysticidal activities were affected by both *Acanthamoeba* strains and the type of eye drop used. Among the four strains, *Acanthamoeba castellanii* Neff was the most

sensitive strain for both stages. This result is in accordance with our previous studies on *Acanthamoeba*, wherein *Acanthamoeba castellanii* Neff was most sensitive to eye drops including Systane Ultra, Optiben and Colircusi Humectante (Sifaoui, I. et al., 2018). TobraDex, Cusimolol were the most active eye drop against all the strain and stages. In a previous work, we have demonstrated the effect of Timolol Sandoz 0.5% to inhibit *Acanthamoeba* spp (Sifaoui, I. et al., 2017). The timolol maleate, present in both Timolol Sandoz and Cusimolol as the active molecule, is a beta blocker that was reported by Hänel et al. (1995) to inhibit Candida albicans via the inhibition of the phospholipase production. In fact, phospholipases could be used as chemotherapy target to prevent *Acanthamoeba*'s penetration and lysis of cell host (Tripathi et al., 2013). As for the TobraDex it contains two active ingredients namely Tobramycin (3 mg/mL) and Dexamethasone (1 mg/mL). At a concentration higher than 250 μg/mL, Tobramycin was reported to inhibit *Acanthamoeba* and thus by inhibiting the protein synthesis (Siddiqui et al., 2016a). As for dexamethasone, could be used to treat inflammation induce by AK, yet as steroid the dexamethasone should be administrated with precaution and for short period, as it was proved to increase *Acanthamoeba* pathogenicity in an *in vivo* model (McClellan et al., 2001; Khan 2006). Colicursi in other hand, was effective only on trophozoite stage of all strains and the cyst stage of *Ac. castellanii* Neff; Bergmanson et al. (2011) have reported that under conditions of high osmolality, (NaCL at a concentration of 5%) the proportion of viable *A. polyphaga* and *A. castellanii* Neff trophozoites was lower than 5% (Bergmanson et al., 2011), nerveless, this concentration was ineffective to inhibit the cyst form of both *Acanthamoeba*.

Program cell death is a type of cell self-death program which cells undergo during development in order to eliminate damages cells (Pistritto et al., 2016). Actually, several types of PCD have been proposed namely apoptosis, autophagy and necrosis (Menna-Barreto 2019). Recently, several studies have proved that parasitic protozoa may undergo a cell death pathway analogous to the process described as apoptosis in metazoan called apoptosis-like process. It has been reported in different protozoa including *Leishmania, Trypanosoma* and has been previously reported in our laboratory in *Acanthamoeba* genus (Sifaoui et al., 2017; Cartuche et al., 2019). The process of PCD in protists appears to share several cellular features with apoptosis in multicellular organisms including chromatin condensation (Debrabant et al., 2003), cell shrinkage (Debrabant et al., 2003) and loss of mitochondrial membrane potential (Kaczanowski et al., 2011), over production of reactive oxygen species among other phenomena (Welburn et al., 2006).

In the present study, the effect of the most active eye drops (TobraDex and Cusimolol) on *Acanthamoeba* spp was studied by examining several morphological features associated with apoptosis like process. After 24 h of incubation with 10% of the eye drops all the apoptosis-like feature evaluated have been accentuated namely chromatin condensation, decrease in ATP and mitochondria membrane potential and increase in the intracellular reactive oxygen species. In a previous work, we reported that Timolol Sandoz eye drop could induce the same clinical changes cited above in *Acanthamoeba castellanii* Neff (Sifaoui, I. et al., 2017). The timolol, active ingredient of Cusimolol and Timolol Sandoz is widely used to treat glaucoma. Several studies have shown that timolol could modulate the calcium pump affecting the Ca^{2+} homeostasis (Wang et al., 2019). In several cell types, the increase in intracellular calcium level has been associated with PCD (Koutsogiannis et al., 2019). In *Acanthamoeba*, Koutsogiannis et al. (2019) have found that G418 induced a PCD-like process via escalate of intracellular calcium concentration followed by a mitochondria malfunction and the release of cytochrome C (Koutsogiannis et al., 2019). Calcium pump could be interesting target in order to eliminate *Acanthamoeba*, due to the fact that calcium channels play a critical role in the viability, motility and the encystation process of *Acanthamoeba* (Siddiqui et al., 2016b).

The aminoglycoside antibiotics including tobramycin, paromomycin, neomycin, neosporin are known to inhibit bacterial growth through the inhibition of protein synthesis. Beside antibacterial effects, He et al. (2000) tobramycin was found to inhibit cell proliferation by regulating the G2/M transition in cancer cell models, including A549 and HeLa-Caco2 cell lines (He et al., 2020). Among studied aminoglycoside, G418 has been reported to induce apoptosis-like process in *Acanthamoeba* (Koutsogiannis et al., 2019) and *Entamoeba histolytica* (Villalba et al., 2007). Both active ingredients, timolol and tobramycin could induce program cell death like process in *Acanthamoeba* via the mitochondria pathway.

5. Conclusion

In summary, TobraDex, Cusimolol eye drops could inhibit *Acanthamoeba*'s trophozoite and cyst at a concentration lower than 5%. Furthermore, treated amoebae showed signs of apoptosis-like induction through the mitochondrial pathway. Herein, the amoebicidal effect as secondary pharmacological effect of both cited eye drops was conducted against four different strains of *Acanthamoeba*. The current eye drops could be used as part of AK therapy protocol Nevertheless, further studies including the effects of both drgus in mitochondrial respiratory chain complexes, in antioxidant enzyme systems and in AK Patients could be interested to confirm the *in vitro* effects.

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Note

Supplementary data associated with this article.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.02.007.

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