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In vitro evaluation of commercial foam Belcils® on *Acanthamoeba* spp

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ABSTRACT

Interest in periocular (eyelid and eyelashes margins) hygiene has attracted attention recently and a growing number of commercials eye cleanser and shampoos have been marketed. In the present study, a particular eye cleanser foam, Belcils® has been tested against trophozoites and cysts on the facultative pathogen *Acanthamoeba*. Viability was tested by the alamarBlue™ method and the foam was tested for the induction of programmed cell death in order to explore its mode of action. We found that a 1% solution of the foam eliminated both trophozoite and cyst stage of *Acanthamoeba* spp. After 90 min of incubation, Belcils® induced, DNA condensation, collapse in the mitochondrial membrane potential and reduction of the ATP level production in *Acanthamoeba*. We conclude that the foam destroys the cells by the induction of an apoptosis-like process. The current eye cleanser could be used as part of AK therapy protocol and as prevention from AK infections for contact lens users and post-ocular trauma patients.

1. Introduction

Corneal diseases, especially infections keratitis, are one of the major causes of blindness and vision impairment in the world (Bharathi et al., 2009). A multitudes causatives agent of microbial keratitis are reported across the world, with bacteria as the leader followed by fungi, Virus and protozoa (Oliveira-Ferreira et al., 2019). Due to the lack of diagnostic tools, the first case of keratitis caused by *Acanthamoeba* has been relatively recently reported in 1973 (Illingworth and Cook, 1998). In the past, this infection has been mainly associated with corneal trauma but in the last decades, it has been associated with contact lenses and its incidence has been increasing due to the rise in contact lens use (Illingworth and Cook, 1998).

The AK infection is known for nonspecific symptoms, early stage infection, as most patients suffers from photophobia, pain, and watering (Illingworth et al., 1995). At a late stage of infection, signs of AK infection will include a "ring-like" stromal infiltrate, stromal thinning and corneal perforation (Szentmáry et al., 2019; Somani et al., 2019). Several reports have listed the risk factors of AK infection in contact lens users including contact lens's materials, the presence or absence of disinfection process and the types of disinfection used to clean the contact lenses (Seal et al., 1999). As for the non-contact lens users, the AK infection could be caused by trauma such as, exposure to contaminated water and the use of traditional eye medicine (Sharma et al., 2000).

In some cases, AK could be generated by both trauma and medical contact lens use. Rama et al. (2008) have reported an AK case due to the use of tap water for face cleaning after undergoing a corneal crosslinking therapy. In fact, the patient was unaware that he was wearing a bandage contact lens and kept cleaning his face and eyelids with tap water (Rama et al., 2009). Those findings were confirmed with other report. Soner Koltas et al. (2015) have confirmed that trauma and exposure to contaminated water were major risk factors to get an AK infection for non-contact lenses users. Moreover, they have demonstrated the presence of the same genotyping of *Acanthamoeba* from AK cases in non-contact lens wearers and in their corresponding domestic tap water

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Table 1
Amoebicidal activities of Belcils® foam 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
IC_{50} Belcils®	0.16 ± 0.02	0.21 ± 0.01[a]	0.21 ± 0.07	0.59 ± 0.01[b]	0.14 ± 0.01	0.56 ± 0.01[b]	0.17 ± 0.01	4.70 ± 0.07[c]

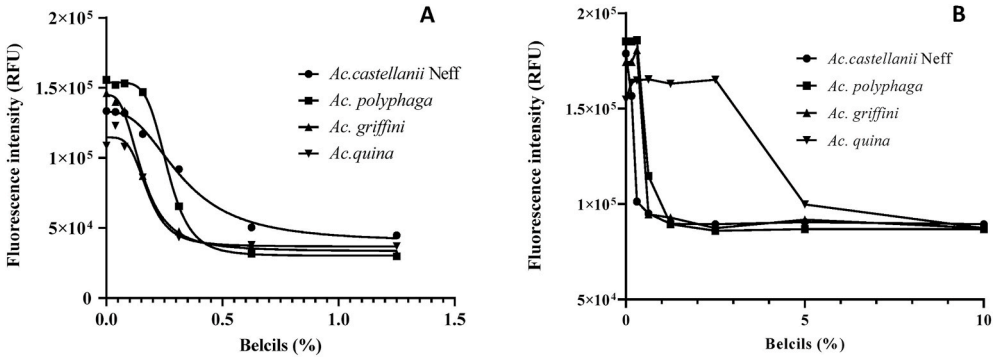

Fig. 1. Standard curve analysis for IC_{50} and IC_{90} calculation using increasing doses of Belcils® determined by the alamarBlue™ assay. A: trophocidal activity, B: Cysticidal activity.

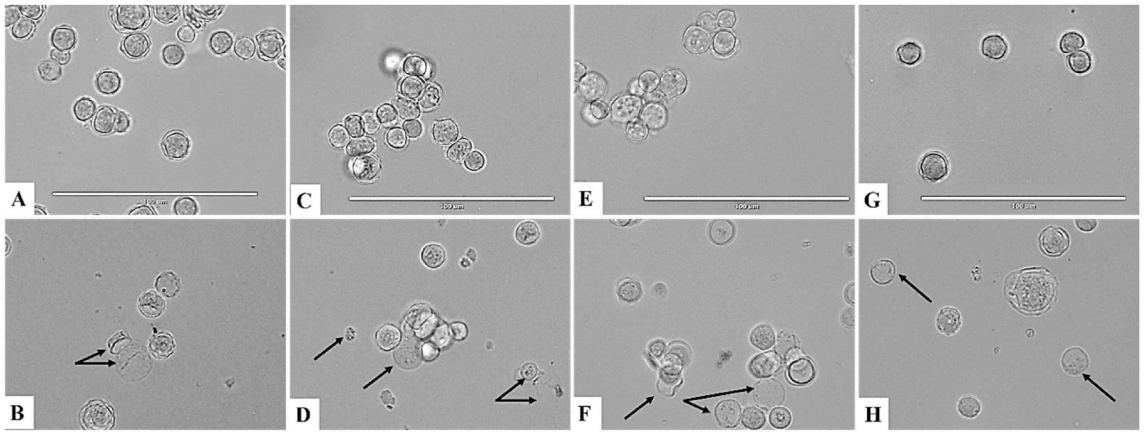

Fig. 2. Effect of the Belcils® foam at 10% on *Acanthamoeba* spp. (A) *Acanthamoeba castellanii* Neff (A); *Acanthamoeba polyphaga* (B); *Acanthamoeba quina* (C); *Acanthamoeba griffini* (D). Black arrowheads indicate shrunken and unviable cells. Images (40X) are representative of the cell population observed in the performed experiments. Images were obtained using an EVOS FL Cell Imaging System AMF4300, Life Technologies, USA.

(Koltas et al., 2015). Most of those cases were cited in developing countries and thus due to the lack of water treatment process and the presence of water storage tanks (Koltas et al., 2015).

For those reasons a good eyelid hygiene is crucial for anterior blepharitis treatment but as well to prevent any other pathologies as AK by eliminating microorganisms namely bacteria (Benitez-del-Castillo, 2012) and *Acanthamoeba*. Consequently, the eye care practitioners (ECPs) emphasized the importance of eyelid hygiene in ocular and tear film health (Bitton et al., 2019). Several eye cleansers contain in addition to a mild cleansing surfactant an antimicrobial component to prevent the formation of biofilm on the lashes and eyelid margin (Bitton et al., 2019). In a previous work, we have demonstrated *in vitro* the efficacy of Naviblef® Daily Care foam to inhibit *Acanthamoeba* spp. (Reyes-Batlle et al., 2019). In the present study, the effect of Belcils® as foam was evaluated against *Acanthamoeba* spp. and an elucidation of its action mode during the time was conducted against *Acanthamoeba castellanii* Neff.

2. Material and methods

2.1. Chemical

In the present study the Belcils® foam, an eye daily care cleaning, was selected to evaluate its anti-*Acanthamoeba in vitro* activity. The foam is principally composed of Bisabolol and Bardana extract.

2.2. Acanthamoeba strains

The trophocidal activity of Belcils® foam 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). 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).

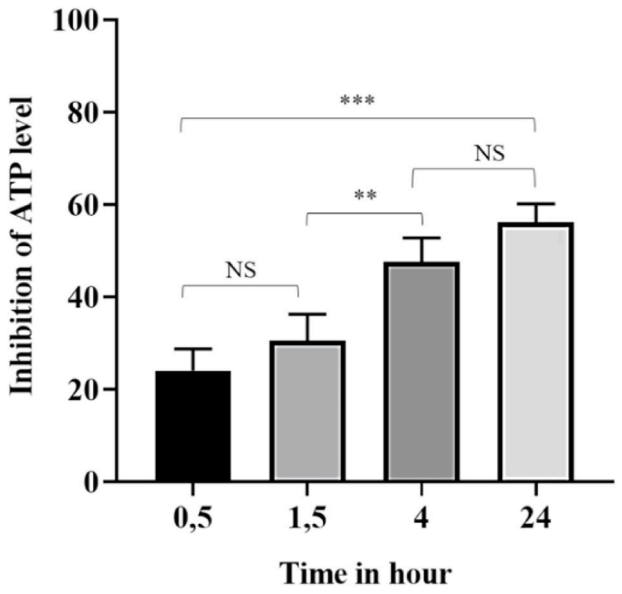

Fig. 3. The effect of Belcils® foam at IC_{90} on *Acanthamoeba castellanii* Neff on the ATP content, using CellTiter-Glo® luminescent cell viability assay during incubation time. Results are representing in percentage relative to the negative control. Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3); NS non-significant; **p < 0.01; ***p < 0.001 significance differences when comparing the different mean values.

2.3. In vitro effect against the trophozoite stage of acanthamoeba

The anti-*Acanthamoeba* activity of the Belcils® solution were determined by the alamarBlue™ assay as previously described (Martín-Navarro et al., 2008; Sifaoui et al., 2017a, 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^{-1}. 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 (Bioresource, Europe, Nivelles, Belgium) was added into each well at an amount equal to 10% of the medium volume. The plates were then incubated for 96 h at 28 °C with a slight agitation and the emitted fluorescence was measured 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 at 144 h and confirmed visually by inverted microscopy. 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 MgSO4·7H2O, 0.4 mM CaCl2·2H2O, 1 mM NaHCO3, 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 foam was made in PYG. The *in vitro* susceptibility assay was performed in sterile 96-well microtiter plates (Corning™). 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 7days 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 (Biosource, Europe, Nivelles, Belgium) was placed into each well, and the plates were then incubated for 144 h at 28 °C with slight agitation and the emitted fluorescence was periodically examined 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 drug on the ATP inhibition was evaluated by incubating (10^5) of cells/ml with the previously calculated IC_{90} of the present solution (Sifaoui et al., 2018).

2.6. Double-stain assay for programmed cell death determination

A double-stain apoptosis detection kit Chromatin Condensation/Dead Cell Apoptosis Kit with Hoechst 33342 and PI (Invitrogen, Madrid, Spain) and an EVOS FL Cell Imaging System AMF4300 (Life Technologies, USA) were used. The experiment was carried out by following the manufacturer's recommendations, and 10^5 cells/well were incubated in a 96-well plate for 0.5, 1.5, 4 and 24 h with the previously calculated IC_{90}. The double-staining pattern allows the identification of three groups in a cellular population: live cells will show only a low level of fluorescence, cells undergoing PCD will show a higher level of blue fluorescence (as chromatin condenses), and dead cells will show 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 IC_{90} of the eye cleanser for 1.5, 4 and 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, 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 IC_{90} of Belcils® solution for 1.5, 4 and 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, USA. The staining pattern allows the identification of two groups in a cellular population: live cells will show only red fluorescence; cells with low mitochondrial potential, (undergoing PCD) will show 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 1.5, 4 and 24 h with the IC_{90} 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, USA (Cartuche et al., 2019).

2.10. Statistical analysis

All images were analyzed with FIJI software for fluorescence quantification. All data are expressed as the mean ± standard deviation of at

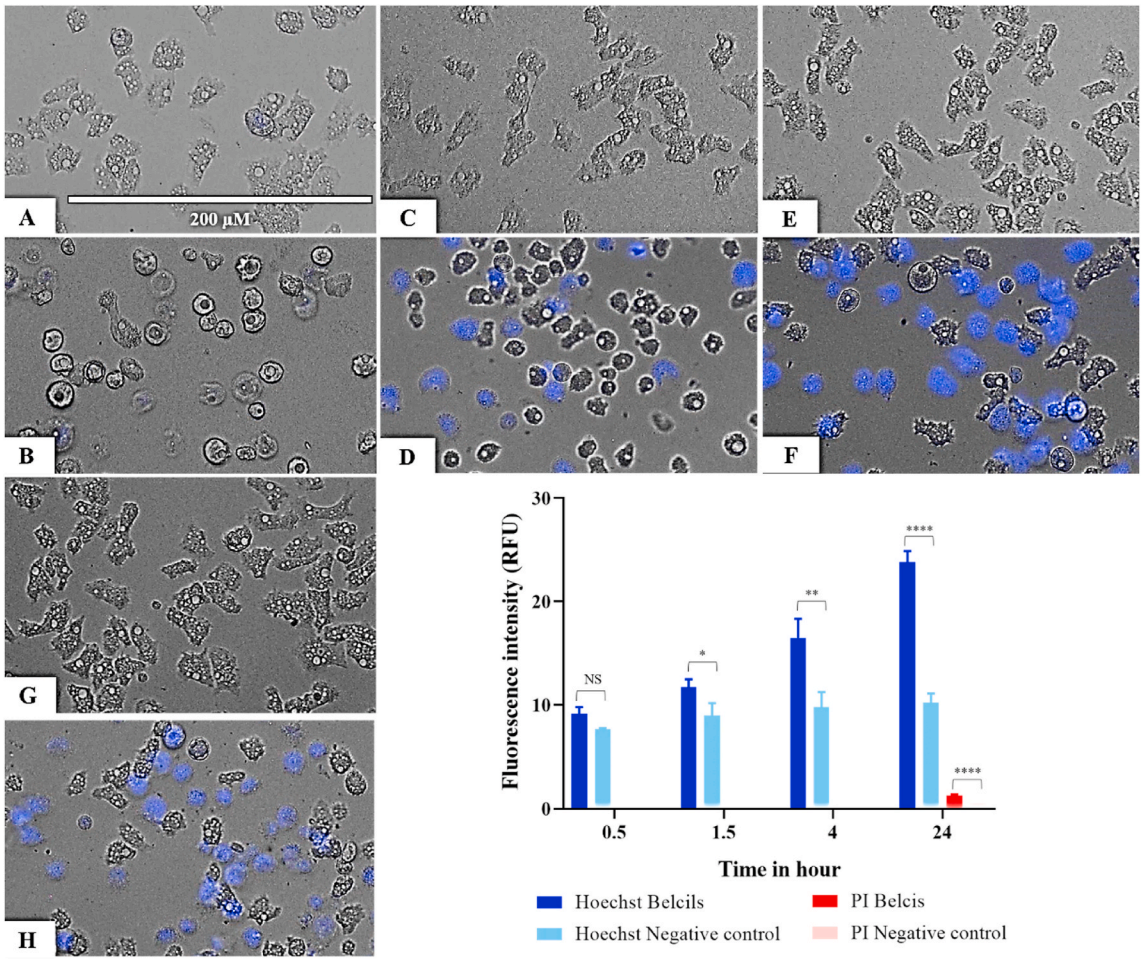

Fig. 4. *Acanthamoeba castellanii* Neff trophozoites incubated with IC_{90} (**B, D, F, H**) of the tested eye cleanser and the evolution of chromatin condensation observed for 30 min (B), 1.5h (D), 4h (F), 24h (H). Hoechst stain is different in control cells (**A, C, E, G**), where uniformly faint-blue nuclei are observed, and in treated cells, where the nuclei are bright blue. Red fluorescence corresponds to the propidium iodide stain. Images (20X) are showing chromatin condensation (blue) in *Acanthamoeba* treated cells. Images (20X) are representative of the cell population observed in the performed experiments. Images were obtained using an EVOS FL Cell Imaging System AMF4300, Life Technologies, USA. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) **p < 0.01; ***p < 0.001; ****p < 0.0001 significance differences when comparing treated cells to negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

least three independent experiments. To highlight the Belcils® effect on *Acanthamoeba* spp. a statistical comparison was conducted using t-test and one-way analysis of variance (ANOVA). All the analysis and the graphics were done by GraphPad Prism version 8.0. Statistical significance was set at $p < 0.05$.

3. Results

3.1. In vitro activity of the Belcils® foam against Acanthamoeba spp

In the present study, Belcils® foam was screened for its activity against four *Acanthamoeba* strains. All IC_{50} values are summarized on Table 1.

In the evaluation of the results, it could be observed that the amoebicidal activity was based on a dependent-dose application (Fig. 1 A and B). Even thought, the eye cleanser used was able to inhibit all the strains with an $IC_{50} < 0.5\%$ (v:v) for trophozoite stage and an $IC_{50} < 5\%$ (v:v) for the cyst stage (Fig. 2), the ANOVA analysis revealed that only the cysticidal activity was significantly affected by the type of strain used with $p < 0{,}0001$. Moreover, the *Acanthamoeba quina* was the most resistant strain while *Acanthamoeba castellanii* Neff was the most sensitive strain.

3.2. Belcils® decreases ATP levels during the time of incubation

We further inspected the effect of eye cleanser on the total ATP levels produced during the time relative to the control. We found out that the foam produced a pronounced inhibition in the total ATP level by 24% after just 30 min of incubation to 56% after 24 h of treatment (Fig. 3).

3.3. Belcils® foam treated amoebae stained positive in the double stain assay after 90 min

After performance of the double staining protocol, ANOVA analysis of the different fluorescence means revealed that the foam at concentrations of IC_{90} could induce chromatin condensation after 90 min. Treated amoebae showed bright-blue stained nuclei after 1.5 h (Fig. 4D) of incubation. While the negative control maintained a stable fluorescence mean, fluorescence of treated cells has increased more than twofold time from 0.5 to 24 h of incubation (Fig. 4). The propidium iodide did stain amoeba only after 24 h, suggesting a late apoptotic stage.

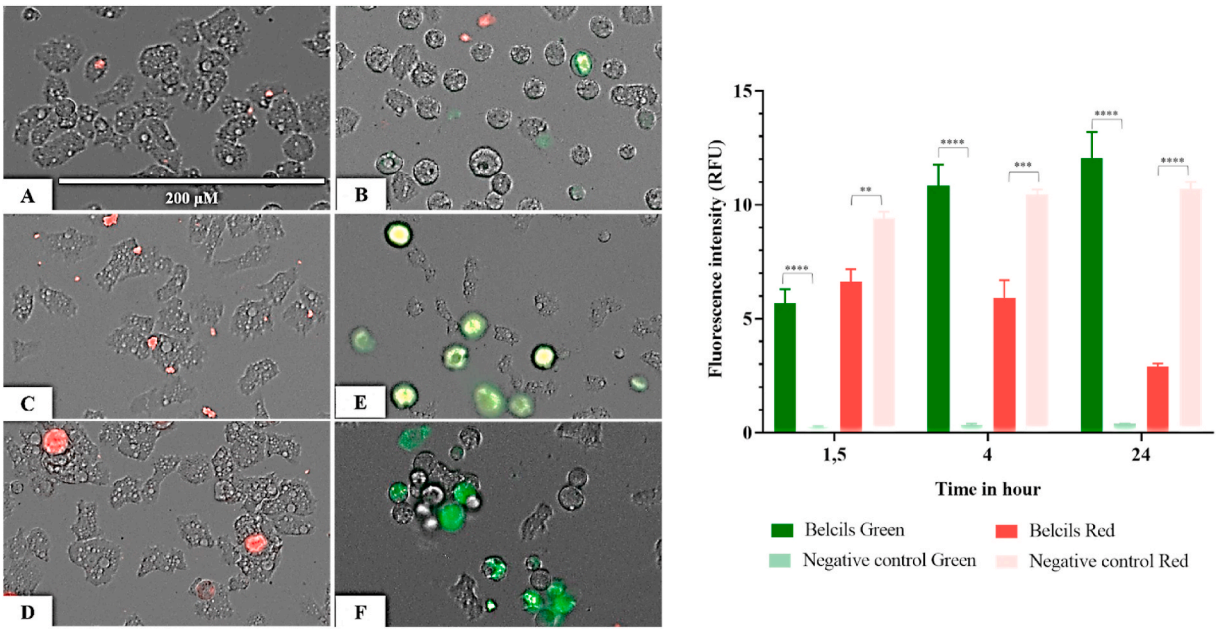

Fig. 5. Images (20X) obtained from EVOS FL Cell Imaging System showing the effect of IC_{90} of Belcils® (B, E, F) on the mitochondrial potential of *Acanthamoeba castellanii* Neff compared to the control (A, C, D) using JC1 kits after 1.5h (A, B); 4h (C, E) and 24h (D, F) of incubation. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) **p < 0.01; ***p < 0.001; ****p < 0.0001 significance differences when comparing treated cells to negative control.

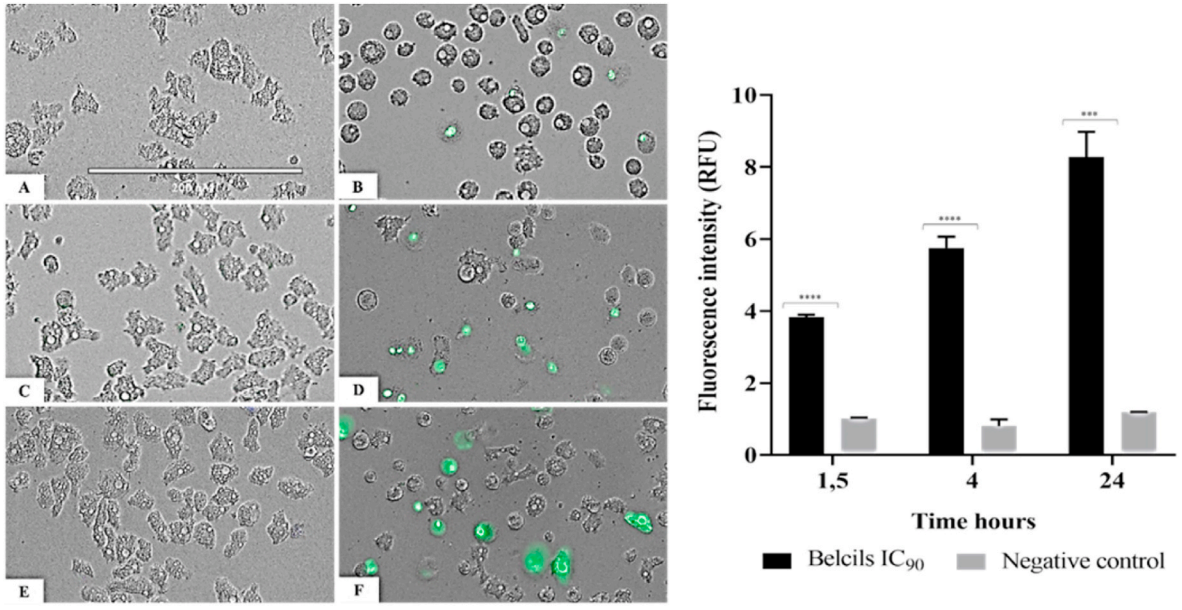

Fig. 6. Images (20X) obtained from EVOS FL Cell Imaging System showing the effect of IC_{90} of Belcils® (B, D, F) on the membrane permeability of *Acanthamoeba castellanii* Neff compared to the control (A, C, E) using Sytox Green dye after 1.5h (A, B); 4h (C, D) and 24h of incubation (E, F). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) **p < 0.01; ***p < 0.001; ****p < 0.0001 significance differences when comparing treated cells to negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Belcils® foam induced the collapse of mitochondrial membrane potential (ΔΨm)

Fig. 5, shows that the eye cleanser could induce the depolarization of the mitochondrial potential since the JC-1 dye remained in the cytoplasm in its monomeric form, shown as green fluorescence. In healthy cells, the dye accumulates in the mitochondria as aggregates and gives a red fluorescence signal (Fig. 5, A, C and D). ANOVA analysis of the different fluorescence means indicated that green and red fluorescence of the treated cells was statically different than the fluorescence emitted by the negative control. Moreover, the green fluorescence enhanced twofold times from 1.5 to 24 h of incubation.

3.5. Belcils® foam caused plasma membrane permeability in treated cells

The possible action of the tested eye cleanser at IC_{90} on

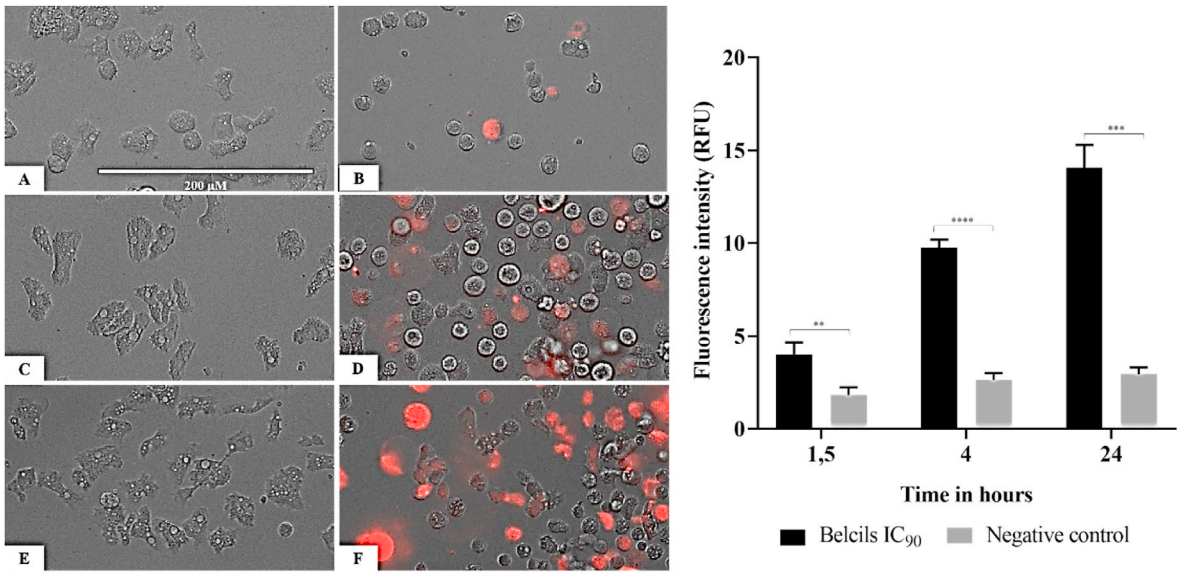

Fig. 7. Images (20X) obtained from EVOS FL Cell Imaging System showing the effect of IC_{90} of Belcils® (B, D, F) on the ROS production of *Acanthamoeba castellanii* Neff compared to the control (A, C, E) after 1.5h (A, B); 4h (C, D) and 24h of incubation (E, F). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) **p < 0.01; ***p < 0.001; ****p < 0.0001 significance differences when comparing treated cells to negative control.

Acanthamoeba castellanii Neff membrane was studied using the fluorescent probe SYTOX Green. This dye 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 (Reyes-Zurita et al., 2013). Our data clearly demonstrated that the membrane damage occurs after 1.5h of treatment (Fig. 6). ANOVA analysis revealed that treated cells emitted higher and significant green fluorescence than the negative control. The signal emitted after 24h was twofold higher than the signal emitted after 1.5h.

3.6. Belcils® foam increases reactive oxygen species (ROS) levels in *Acanthamoeba castellanii neff*

The analysis of the production of Reactive Oxygen Species was conducting using the CellROX Deep Red™ dye. Fig. 7 showed that treatment of *Acanthamoeba castellanii* Neff with the eye cleanser noticeably enhanced the red fluorescence reflecting the increase of ROS intracellular production. A one-way ANOVA analysis was done, and mean fluorescence emitted by the treated cells was higher and significant difference than the negative control. Histogram of the Fig. 7 shows that the fluorescence emitted by treated cells after 24h was three times the signal emitted at 1.5h.

4. Discussion

Recently and due to the increase of infectious eye pathologies namely ocular Blepharitis, infections keratitis among others, the eye health has been advocated and implemented worldwide (Prozesky et al., 2007; Ravilla and Ramasamy, 2012). The number of eye cleansers products have emerged recently as part of the therapy protocol or to prevent new ocular diseases. In a previous work, we have demonstrated the effect of Naviblef® Daily Care foam to inhibit *Acanthamoeba* spp. (Reyes-Batlle et al., 2019). This action could be results of the presence of Tea tree oil (TTA); in fact, for its antiseptic effect against bacteria, fungi and viruses, this component has been widely used in many eye cleanser and eyelid shampoo (Mohammadpour et al., 2019). The IC_{50} of the eye cleanser Belcils® were much lower than the IC_{50} obtained for Naviblef® Daily Care for all the tested *Acanthamoeba* strains. Consequently, the activity of the present foam was much higher than the Naviblef® Daily Care foam and was independent of the *Acanthamoeba* strain used. This effect could be due to the synergic effect of several antiseptic ingredients: Bisabolol and Burdock extract.

In a previous work, we have reported the amoebicidal activity of Bisabolol, main component of chamomile essential oil (Hajaji et al., 2017). The sesquiterpene had a good activity against *Acanthamoeba castellanii* Neff and a low cytotoxicity effect in murine macrophages cells lines. Burdock extract have been used in Chinese traditional medicine to treat diseases such as sore throat and infections namely rashes, boils and several skin pathologies (Chan et al., 2011). Several reports have confirmed the beneficial effects of this extract as anti-inflammatory, anti-tumoral, anti-diabetic among others (Ahangarpour et al., 2017). Furthermore, this extract contain several active ingredients such tannin, arctigenin, eudesmol, caffeic acid and chlorgenic acid among others (Chan et al., 2011). Eudesmol is one of the major components of *Teucrium ramosissimum* essential oil, which have a good amoebicidal activity against *Acanthamoeba castellanii* Neff (Ghazouani et al., 2017). As for chlorogenic acid it has been already reported for its potent amoebicidal activity against Acanthamoeba triangularis (Mahboob et al., 2016). The actual therapy against AK normally involves using 0.02% biguanides and 0.1% diamidines (Elsheikha et al., 2020), however, resistance to these amoebicidal drugs has increased in many of the *Acanthamoeba* genus. Padzik et al. (2018) (Padzik et al., 2018) have reported that *A. polyphaga* was more resistant to chlorhexidine digluconate solution (0.02%) than the environmental *Acanthamoeba* Neff strain. Although, the cysticidal activity was significantly affected by the type of strain used, as *Acanthamoeba quina* was the most resistant strain while *Acanthamoeba castellanii* Neff was the most sensitive strain. The Belcils® was able to eliminate the four tested strain. The recommended use of the present foam is from 2 to 3 times per day, or whenever necessary. Cleaning eyelids and eyelashes several times per day, would eventually prevent the presence of microorganism namely *Acanthamoeba*.

Programmed Cell Death (PCD) is a Cell Death without inflammation. Recently, different phenotypes for PCD have been listed including apoptosis and autophagy (Menna-Barreto, 2019). Moreover, PCD and apoptosis-like process have been reported in different protozoa namely *Leishmania, Trypanosom* and has been previously reported in our laboratory in *Acanthamoeba* genus (Cartuche et al., 2019; Sifaoui et al., 2014, 2017; Zeouk et al., 2020). The process of PCD involves several cellular

features such as chromatin condensation, cell shrinkage and loss of mitochondrial potential, over production of reactive oxygen species among other phenomena (Debrabant et al., 2003).

In the present study, Belcils® foam could induce an apoptosis like in *Acanthamoeba castellanii* Neff after 90 min of incubation of cells with 0.6% (v:v) of the foam. During the 24 h of incubation all the apoptosis-like feature tests have been accentuated such as the collapse of mitochondrial potential, the decrease in ATP level, the increase of intracellular ROS level and the chromatin condensation. Among the foam ingredients, we have reported that α-bisabolol was able to induce apoptosis like in *Acanthamoeba castellanii* Neff by affecting the mitochondrial membrane potential, the ATP level and cell's membrane (Hajaji et al., 2017). Arctigenin, a natural lignan, one of major compound of *Arctium lappa* (Burdock) extract was described as anti-proliferative, anti-inflammatory, antioxidant, antiviral agent (Kudou et al., 2013). In addition, Maimaitili et al. (2016) have elucidated the pathway of the antiproliferative effect of this lignan on human glioma cells. The authors have proved that Arctigenin affect the cell cycle through the mitochondrial pathway (Maimaitili et al., 2017). As a result, we could assume that the present foam inhibits *Acanthamoeba* spp. via the mitochondrial pathway.

5. Conclusions

Daily periocular region hygiene typically includes cleaning of eyelids and eyelashes using eye cleanser as part of treatment protocols and/or as Infection prevention. In summary Belcils® foam, an eye cleanser, could inhibit the *Acanthamoeba* spp. proliferation at a concentration less than 5% for cyst and trophozoite stage. Furthermore, foam-treated amoebae showed signs of apoptosis-like induction through the mitochondrial pathway. Consequently, the current eye cleanser could be used as part of AK therapy protocol and as preventing tool for contact lens users and as prevention of AK infections for post-ocular trauma. Nevertheless, further studies including a study effect of Belcils® foam on Lid Hygiene and Eyelash Length in Patients with AK infections and with post-ocular trauma could be interested to confirm the *in vitro* effects and to determine the number of foam application to ensure the disinfection process.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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