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The efficacy of Polihexanide (PHMB) eye drops against *Acanthamoeba polyphaga* investigated by an ATP-bioluminescence assay and a rat model of keratitis

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PURPOSE

Acanthamoeba keratitis (AK) is a rare but severe infectious disease caused by *Acanthamoeba* spp. a ubiquitous free living protozoan. The incidence is uncertain but probably <500 cases/year based on the EU population of about 500 million, and as low as 0.15-0.18 per million in the USA (Acharya *et al*., 2007). In the absence of treatment, the disease progresses to blindness as a result of corneal vascularisation and scarring or corneal perforation. The current medical treatments for this rare and severe disease have not been evaluated in preclinical studies. The most widely used include unlicensed antiseptic drugs employed alone or in combinations, most commonly a biguanide (e.g. Polihexanide, PHMB) either as monotherapy or combined with a diamidine (Propamidine or Hexamidine). PHMB 0.02% alone or in combination has shown efficacy in a clinical retrospective study (Papa *et al*., IOVS 2015). However, the concentration of this drug, and the treatment schedules, has been developed empirically. Purpose of our study is to assess the efficacy of PHMB at different concentrations against *Acanthamoeba polyphaga* using *in vitro* and *in vivo* test systems.

MATERIAL AND METHODS

In vitro

Trophozoites of *Acanthamoeba polyphaga* ATCC 50495 were cultured in peptone yeast extract-glucose (PYG) medium at 25°C. Then 5×10^6 amoebae/ml were collected by centrifugation for 10 min at 150×g and induced to encyst by using Neff's constant-pH medium at 31°C at least for 2 weeks. A linear correlation curve among amoebae number (haemocytometric count) and Relative Light Unit (RLU) was produced. ATP-bioluminescence assay (Pallcheck® System; Fig. 1) was validated with respect to the traditional count method. Efficacy of 1/10, 1/100 and 1/1000 dilutions of PHMB (0.02%, 0.04%, 0.06% and 0.08%) was tested by using ATP-bioluminescence assay. Killing kinetic curves were created using $5x10^4$ cysts suspension exposed for 30 min, 1, 3 and 7 hours to 1/10 and 1/100 dilutions of the above selected PHMB concentrations. Statistical analysis was conducted by two-way Anova plus Bonferroni post test.

Fig. 1. Pallchek™ System

In vivo

Five weeks old Sprague-Dawley male rats were injected in the left cornea stromal layer with 10^4 trophozoites (*Acanthamoeba polyphaga*, ATCC 50495). A subconjunctival injection of 0.57 mg long-acting betamethasone was administred and clinical examination was performed weekly. Rats inoculated with *Acanthamoeba polyphaga* were divided into 6 groups and topically treated 4 times a day with PHMB (0.02%, 0.04%, 0.06% and 0.08%) or a combination of PHMB and Propamidine (0.02% and 0.1%, respectively). Control animals were treated similarly with PHMB vehicle. Clinical infection was defined by corneal oedema and/or infiltration (associated or not with neovascularisation). Keratitis opacity lesions were scored from grade 0 (no lesions) to grade 3 (corneal opacification obscuring iris vessels details) according to Polat *et al*., 2014 (Fig. 2 and 3). At day 28, the rats were sacrificed. Corneal scrapings were performed for bacterial and parasitological cultures and real-time PCR analyses (Qvarnström *et al*., 2006). Paraffin-embedded corneas were analysed after hematoxylin-eosin and Schiff periodic acid staining. All animal procedures were performed according to the guidelines of the ARVO statement for the "Use of Animals in Ophthalmic and Vision Research". Statistical analysis was performed by estimating the significance of Fischer's exact tests.

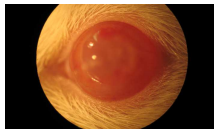

Fig. 2. Grade 1 rat keratitis lesion

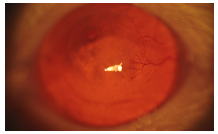

Fig. 3. Grade 3 rat keratitis lesion

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RESULTS

In vitro efficacy

ATP-bioluminescence assay showed that only 1/10 and 1/100 dilutions of the selected PHMB concentrations (0.08%, 0.06%, 0.04%, 0.02%) reduced cysts viability of 80% and 50 % respectively (Fig. 4). To determine the most effective PHMB solution a killing curve was generated with 100-fold diluted of the selected PHMB concentrations. The curve (Fig. 5) showed that reduction on cysts viability at 3h was 60% with PHMB 0.04%, 0.06% and 0.08% whereas PHMB 0.02% 100-fold diluted proved to be the least effective with 40% reduction on cysts viability. Multiple comparative analysis is shown in Table1.

Fig.4. Viability of *Acanthamoeba* cysts after 1-hour incubation (y-axis). PHMB concentrations (■0.02%, ■ 0.04%, ■ 0.06%, ■0.08%), 10-100-1000 fold diluted (x-axis).

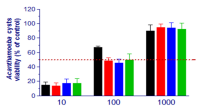

Fig.5 Killing kinetics of PHMB concentrations (■0.02%, ■0.04%, ■ 0.06%, ■0.08%),100-fold diluted, on *Acanthamoeba* cysts.

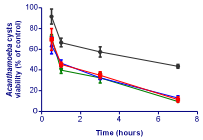

Table 1. Two way-Anova plus Bonferroni post test (**p< 0.01; ***p< 0.001; ****p< 0.0001)

[PHMB]	0.04%/100				0.06%/100				0.08%/100			
	0.5h	1h	3h	7h	0.5h	1h	3h	7h	0.5h	1h	3h	7h
0.02%/100	****	****	****	****	****	****	****	****	****	****	****	****
0.04%/100					ns				ns			
0.06%/100	ns								ns			
0.08%/100	ns				ns							

In vivo efficacy

Our keratitis model showed that PHMB 0.04% and 0.06% significantly prevented corneal lesions worsening between day 14 and day 28 compared to the control group. We could observe the same tendency for PHMB 0.08%, although was not significantly. PHMB 0.02% also in combination with Propamidine is less effective than the other PHMB concentrations (Table 2). PHMB 0.04%, 0.06% and 0.08% significantly decreased cultures/PCR and/or histology positivity compared to the control group (Table 3).

Table 2. Evaluation of the clinical efficacy of corneal PHMB treatment on the course of *Acanthamoeba polyphaga* keratitis in rats.

Group	D28 Grade 0 [a]	D28 Grade 1	D28 Grade 2	D28 Grade 3	D 14- 28 grade worsening[b]
Control (n=12)	0/12	1/12	4/12	7/12	9/12
PHMB 0.02% (n=6)	0/6	0/6	4/6	2/6	2/6
PHMB 0.04% (n=11)	3/11	1/11	4/11	3/11	0/11**
PHMB 0.06% (n=11)	2/11	2/11	5/11	2/11	2/11*
PHMB 0.08% (n=11)	2/11	1/11	5/11	3/11	4/11
PHMB 0.02% + Propamidine (n=11)	1/11	1/11	6/11	3/11	7/11

a Ratio of animals without D28 corneal lesions (Full recovery)
b Ratio of animals with differences between D28 and D14 corneal lesions grades > 0
Asterisks indicate significant differences with the control group: * p<0.05 compared with the control group; ** p<0.01 compared with the control group

Table 3. Evaluation of the parasitological efficacy of corneal PHMB treatment on the course of *Acanthamoeba polyphaga* keratitis in rats.

Group	Histology[a]	Culture[b]	PCR[c]
Control (n=12)	11/12	9/12	10/12
PHMB 0.02% (n=6)	6/6	4/6	6/6
PHMB 0.04% (n=11)	4/11**	2/11*	2/11**
PHMB 0.06% (n=11)	5/11*	4/11	5/11
PHMB 0.08% (n=11)	5/11*	4/11	5/11
PHMB 0.02% + Propamidine (n=11)	5/11*	4/11	4/11*

a Ratio of animals with present *Acanthamoeba polyphaga* forms in corneal tissues
b Ratio of animals with *Acanthamoeba polyphaga* positive corneal scraping cultures
c Ratio of animals with *Acanthamoeba polyphaga* positive corneal scraping PCR.
In all columns, asterisks indicate significant differences with the control group:
* p<0.05 compared with the control group ; ** p<0.01 compared with the control group

CONCLUSIONS

Our *in vitro* and *in vivo* tests systems have provided the same results as concerning the identification of 0.02% PHMB as the least effective formulation against *Acanthamoeba polyphaga*. In addition, *in vivo* data confirm that monotherapy with PHMB solutions at concentration equal or more than 0.04 % seems to be effective against *Acanthamoeba polyphaga*. Further investigations are needed to improve the discriminating power of these test systems. Nevertheless, our experimental approach represents a very useful preliminary tool for selecting the most appropriate PHMB concentration to be tested as drug candidate for clinical use.

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