


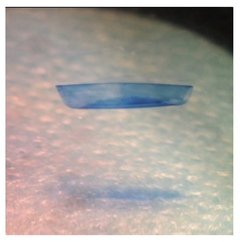
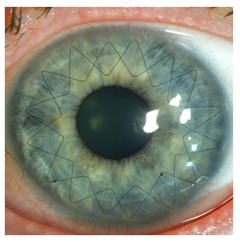


The effect of antiamoebic agents and chlorin e6-PDT on *Acanthamoeba castellani* trophozoites and cysts *in vitro*

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Background and Purpose

Acanthamoeba infection in immunocompromised patients may attack the central nervous system and immunocompetent subjects may develelop Acanthamoeba keratitis (AK). AK incidences have been reported with increasing frequency worldwide [1-3], particularly in contact lens wearers [4, 5].
AK is a serious, sight-threatening disease, in which patients need long-term treatment. However, up-to date, no standardized treatment is available. All antiamoebic drugs in clinical use are off-label.
In order to optimize clinical treatment of AK, the purpose of this study was to analyze the concentration dependent effect of biguanides (polyhexamethylen biguanid (PHMB), chlorhexidine (CH)); diamidines (hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD)); natamycin (NM); miltefosine (MF); povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) [6-8] on *Acanthamoeba castellani* trophozoites and cysts, *in vitro.*

Materials and Methods

Acanthamoeba castellani 1BU strain was cultured in 712 peptone-yeast extract-glucose (PYG) medium. Thereafter, trophozoites or cysts were cultured in 0,005-0.02% PHMB, CH or 0.25-0.1% HD, PD, DD, or 5% NM or 0.001625-0.0065% MF or 0.25-1% PVPI containing PYG medium for 2 hours or underwent Chlorin e6-PDT (**Table 1**). Then, the percentage of dead trophozoites was determined by CytoTox 96® Non-Radioactive Cytotoxicity assay and trypan blue staining, and those of dead cysts using trypan blue staining. Treated cysts were also inoculated on non-nutrient agar *Eshericia coli* plates [9,10] and were observed for 5 weeks.

Table 1. Antiamoebic agent concentrations in our experiments.

Agents	Maximum	Half of maximum	Quarter of maximum
PHMB	0.02% (1079µM)	0.01% (539.5µM)	0.005% (269.75µM)
CH	0.02% (396µM)	0.01% (198µM)	0.005% (99µM)
HD	0.1% (1648µM)	0.05% (824µM)	0.025% (412µM)
PD	0.1% (1771µM)	0.05% (885.5µM)	0.025% (442.75µM)
DD	0.1% (1384µM)	0.05% (692µM)	0.025% (346µM)
NM	5% (75106µM)	2.5% (37553µM)	1.25% (18776.5µM)
MF	0.0065% (160µM)	0.00325% (80µM)	0.001625% (40µM)
PVPI	1% (27401µM)	0.5% (13700.5µM)	0.25% (6850.25µM)
Ce6	0.0038% (64µM)	0.0019% (128µM)	0.00095% (256µM)

Results

All concentrations of different antiamoebic agents had a significant cytotoxic effect on AK trophozoites and cysts ($p<0.05$), except 0.02% PHMB or CH, for trophozoites, and 0.005% CH or Ce6-PDT for cysts, using trypan blue assay (**Figures 1-3**).
Observing the agar plates, PHMB, CH, HD, PD, NM and PVPI led to morphological changes of *Acanthamoeba* trophozoites, which could not form cysts again within 5 weeks (**Figure 4**). The strange-shaped structures appeared after 24-72 hours, and could move out from the center to the peripheral area of the plate. Some of them had double wall (like cysts), with discontinous outer wall (**Figure 5**).
DD and MF treated cysts could excyst and later encyst again (**Figure 4**).

Conclusions

In vitro analysis of treatment efficacy of different antiamoebic agents, especially the non-nutrient agar *Eschericia coli* plate assay may provide us information on specific treatment of different *Acanthamoeba* strains.
1BU *Acanthamoeba castellani* was more vulnerable to PHMB, CH, HD, PD, NM and PVPI treatment than to DD and MF. However, none of these agents could completely eradicate trophozoites and cysts.

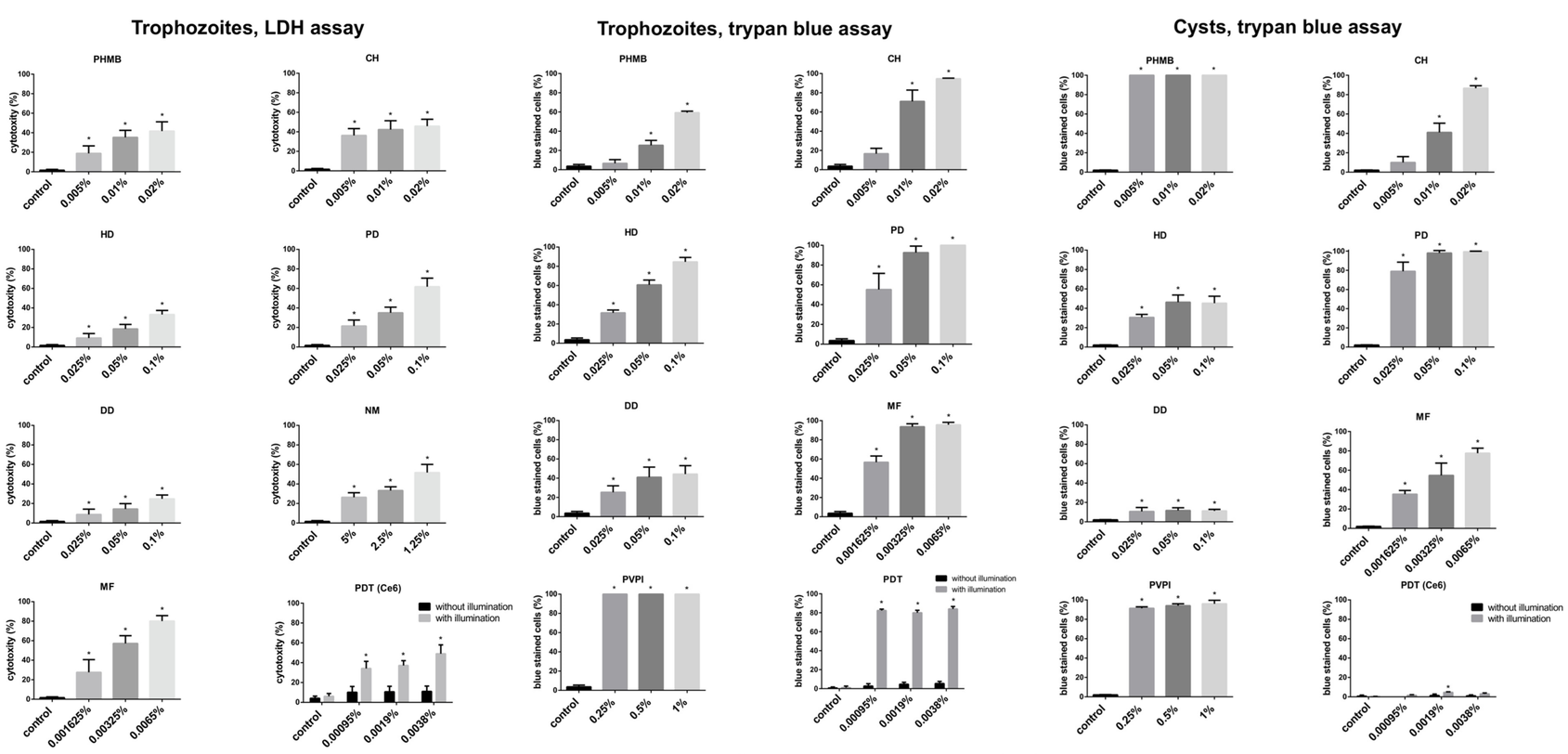

Figure 1. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB), chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), natamycin (NM), miltefosine (MF), and chlorin e6 photodynamic therapy (PDT) on 1BU trophozoites (***LDH assay;*** n=5).
PHMB, CH, HD, PD, DD, NM, MF, and chlorin e6-PDT increased LDH activity significantly (*) in trophozoite cultures ($P<0.01$), compared to controls. The use of Ce6 without illumination did not change LDH activity ($P=0.96$), compared to Ce6 with illumination.

Figure 2. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB), chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), miltefosine (MF), povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on 1BU trophozoites (***trypan blue*** assay; n=3).
All used drugs increased percentage of trypan blue stained 1BU *trophozoites* significantly (*) ($P<0.01$), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU trophozoites significantly ($P\leq0.02$) compared to controls, except 0.005 % PHMB ($P=0.59$), 0.005 % CH ($P= 0.10$) and Ce6 without illuminaton ($P=0.99$).

Figure 3. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB), chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), miltefosine (MF), povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on 1BU cysts (***trypan blue*** assay; n=3).
All used drugs increased percentage of trypan blue stained 1BU *cysts* significantly (*) ($P<0.01$), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU cysts significantly ($P<0.01$) compared to controls, except 0.005 % CH ($P=0.29$), Ce6 without illumination ($P=0.94$) and 0.00095% or 0.0038% Ce6 with illumination ($P=0.14$; $P=0.06$).

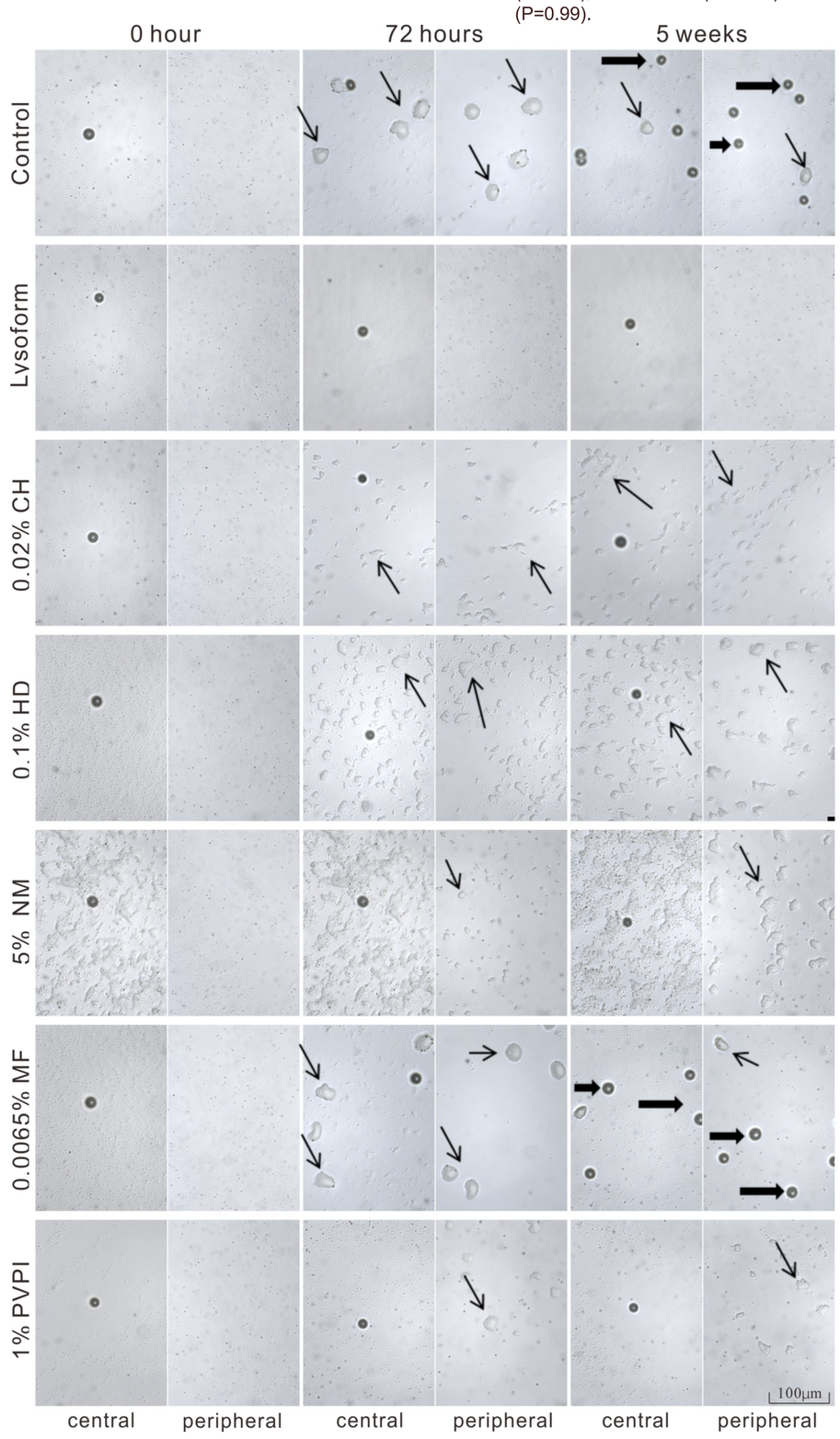

Figure 4. Images of the non-nutrient agar *E. coli* plate assay (following inocuation of cysts), at the center and periphery of the plates at three different time-points.
In lysoform treated positive control group, no trophozoites or fresh cysts emerged during follow-up.
In control and 0.0065% MF groups, normal-shaphed fresh trophozoites appeared from 24 hours (see central and periphreal areas at 72 hours and 5 weeks, arrows) and the trophozoites could move out from the central area of the plate (see peripheral images at 72 hours and 5 weeks, arrows). In these groups, encystment happened again from 1 week (see central and peripheral images at 5 weeks, bold arrows).
In 0.02% CH, 0.1% HD and 1% PVPI groups, we observe strange-shaped structures after 24-72 hours (see central and peripheral images at 72 hours and 5 weeks, arrows), which could move out from the central to the peripheral area of the plate (see peripheral images at 72 hours and 5 weeks, arrows). No fresh round cysts appeared during follow-up. In 1% PVPI group, the strange-shaped structures could only be seen at the peripheral area of the plate (arrows).
At the 5% NM images we also see the „natamycin-dust", which made differentiation of NM and acanthamoeba difficult. There were no new cysts during 5 weeks (see peripheral images at 72 hours and 5 weeks). However, some spherical structures with discontinous outer wall could be observed (arrows).

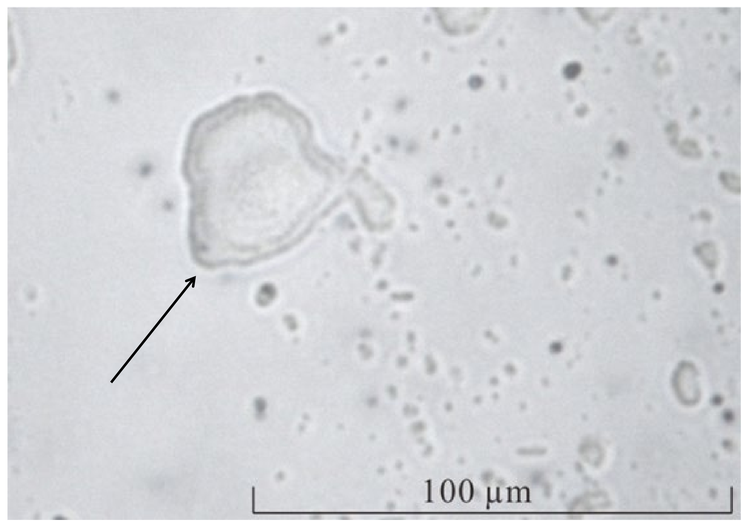

Figure 5. In 0.02% PHMB, 0.1% PD, and 1% PVPI groups, strange-shaped structures appeared after 24-72 hours, which could move out from the center to the peripheral area of the plate. Some of them had double wall (like cysts), with discontinous outer wall. In these groups no new cysts could be observed during 5 weeks of follow-up (arrrow).

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Potentielle Interessenkonflikte: 1. nein, 2. nein, 3. nein, 4. nein, 5. nein

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