In vitro toxicity evaluation against red blood cells of an ophthalmic amphotericin B-microemulsion

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INTRODUCTION AND OBJECTIVES

Ocular fungal infections are an important ophthalmologic problem causing significant ocular morbidity (Carrasco 2011). The pharmacological approach for the management of these infections involves administration of antifungals agents, including amphotericin B (AmB) in its micellar form, Fungizon® (AmB-M). This formulation has been widely used by intravenous, topical, intracameral and intravitreal routes. However, the intravenous administration may cause poor corneal bioavailabitily and severe nephrotoxicity. Indeed, the topical application is reduced in the presence of an intact corneal epithelium (Gratieri 2010, Mazouri 2001, Thomas 2003).

Microemulsion (ME) is a system that contains water and oil coexisting in thermodynamic equillibrium due to the presence of a surfactant film at the oil-in-water interface. They are clear, stable, transparent and isotropic systems and currently have aroused the interest of pharmaceutical scientists because of their capacity to be used for ocular applications (Pestana 2008, Vandamme 2002).

The aim of this work was to evaluate the toxicity against red blood cells of the AmB incorporated into ME (AmB-ME) and to correlate it with the aggregation state of this drug when incorporated into the ME droplet.

MATERIALS AND METHODS

The ME formulation was prepared from a pseudo-ternary phase diagram procedure and had as water phase a phosphate buffer pH 7.4 solution and Lipoid® S100 and as oil phase, Miglyol®812N and Tween®80 (Vandamme 2002). Both water and oil phases were magnetically stirred until complete homogenization. The ME was achieved by addition of the water phase into the oil phase followed by sonication process and ultrasound bath. The drug was incorporated into the system at a concentration of 5.0 mg/mL. Briefly, AmB was dissolved (50 mg/mL) in NaOH solution (1 N) and added to the ME under magnetic stirring. The pH was adjusted to 7.5-8.0 with 1 N HCl solution.

To evaluate the aggregation state, AmB-ME samples were diluted in phosphate buffer pH 7.4 and in the blank ME to yield a concentration of 5 x 10^{-6} mg/mL (5 x 10^{-9} M). The spectrum of these dispersions was recorded at the wavelength from 300 to 450 nm and compared with the ones obtained for the AmB-M in phosphate buffer (pH 7.4) and standard AmB in methanol (AmB-MET) at the same concentration (5 x 10^{-6} mg/mL).

For the *in vitro* toxicity assay, red blood cells (RBC), from human healthy donors were used. Potassium (K^+) and hemoglobin (Hb) leakage from these cells were monitored, respectively, as a measure of acute and chronic toxicity (Araújo 2005).

RESULTS AND DISCUSSION

Clinically, the aggregation state of AmB is directly related to its mechanism of action and toxicity (Araújo 2005). The water soluble monomer is usually associated to a low toxic form of AmB while water-insoluble oligomers have been defined as the toxic one (Silva-Filho 2012). Therefore, depending on its aggregational state, the AmB may present several patterns of activity on mammalian cells (Araújo 2005).

The different AmB aggregation state behavior was measured by the variation on the absorption spectra calculated by the molar extinction coefficients (ε) (Egito 2002). The results revealed that in phosphate buffer the AmB-M presented four characteristics absorption bands, located at 329, 364, 385 and 408 nm. In methanol four bands with decreasing intensities at 406, 383, 363, and 345 nm can be seen. These spectral bands indicate the existence of aggregate states and monomers, respectively (Egito 2002).

A comparison among the UV-visible absorption spectra of AmB-M in phosphate buffer, standard AmB in methanol and AmB-ME diluted in phosphate buffer and in blank ME is presented in Figure 1. As it can be clearly seen on the spectra of AmB-ME diluted in phosphate buffer, the presence of one intense band at 329 nm indicates the formation of self-associated AmB structure. However, the shift of such band from 323 to 329 indicated that the complex between AmB and ME is different from the complex observed by AmB and micelles.

Concerning the Hb leakage, at the concentrations of 50 mg/mL and 5 mg/mL, AmB-ME presented a release of 87.49 ± 0.02 and 1.41 ± 0.05 respectively. For AmB-M, these values changed to 100% and 5.88%, respectively ($p < 0.05$). Therefore, it can be verified that the lethal effect or acute toxicity demonstrated by the cellular lysis was shown to be larger for the AmB-M than for the AmB-ME. This

result suggests that this behavior could be associated to the new complex of self-associated AmB structures, which changed the toxicity profile when compared to AmB-M (Figure 2a) (Damasceno 2011).

Concerning K^+ leakage, all samples of AmB-M and AmB-ME presented a similar profile with nosignificant difference between them. However, in RBCs the K^+ ions inside the cells can be released by any disturbance in the cell membrane that generatespores, which enables the output of K^+ with no release of Hb. In fact, Hb released occurs only after the total disruption of the membrane barrier (Figure 2b) (Silva-Filho 2012).

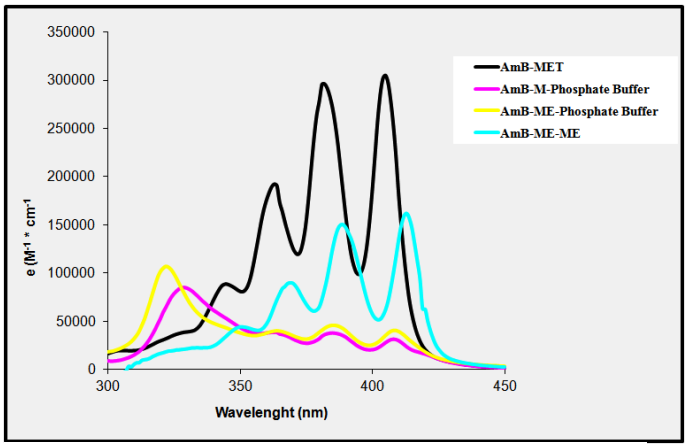
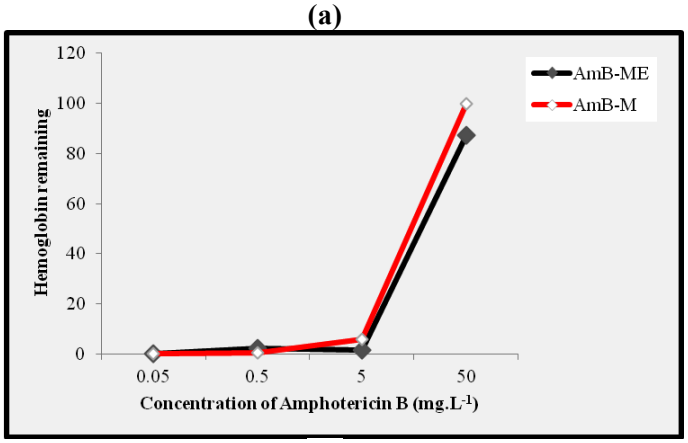


Figure 1. UV-visible absorption spectra of different AmB systems

(a)



(b)

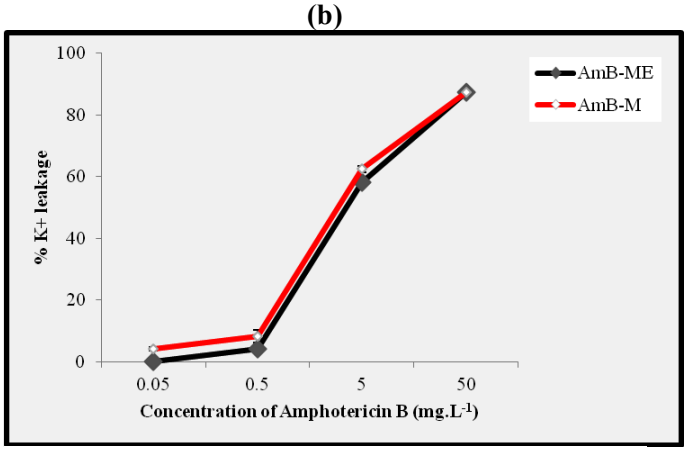


Figure 2. Profile of cytotoxicity of AmB-ME on RBCs. (a) Hb remaining and (b) K^+ leakage

CONCLUSION

These results show that the AmB-ME have a different pattern of toxicity when compared to the AmB-M. The developed AmB-ME can be, therefore, considered as a new alternative for clinical use against susceptibilty fungal cells in ocular fungal infections.

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