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Note

Influence of different buffers (HEPES/MOPS) on keratinocyte cell viability and microbial growth

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ABSTRACT

This study assessed the effect of the buffers 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(N-morpholino) propanesulfonic acid (MOPS) on keratinocyte cell viability and microbial growth. It was observed that RPMI buffered with HEPES, supplemented with L-glutamine and sodium bicarbonate, can be used as a more suitable medium to promote co-culture.

Studies of co-culture with keratinocyte cells and microorganisms have been fundamentally important in assessing the virulence and cytotoxicity of biofilms. The loss of keratinocyte cell viability when cells are exposed to biofilms of *Staphylococcus aureus* can be observed after 3 h of exposure (Kirker et al., 2009). The co-culture of *Candida albicans* and oral epithelium cells stimulates signaling pathways that promote cell death (Villar and Zhao, 2010).

The culture medium used for biofilm growth should not interfere with cell viability. Although RPMI (Roswell Park Memorial Institute) buffered with MOPS has been used, the concentration of MOPS (Zago et al., 2015) is greater than that permitted for cell cultures (Eagle, 1971), and could compromise co-culture studies. As an alternative, HEPES could be used for biofilm formation of *C. albicans* and *S. aureus* (Peters et al., 2010). The present research compared the effect of RPMI buffered with HEPES and MOPS on keratinocyte cell viability and microbial growth.

C. albicans SC5314 and *S. aureus* ATCC25923 microorganisms were used to produce single and dual species biofilms, in accordance with the methodology described by Zago et al. (2015). To prepare the yeast and bacteria pre-inoculum, a loop full of agar stock cultures was transferred to 10 mL of Yeast Nitrogen Base broth (YNB — Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and Tryptic Soy Broth (TSB — Acumedia Manufactures Inc., Baltimore, MD, USA), respectively, and incubated at 37 °C overnight. Thereafter, the dilution of the inoculum was performed and cultures were incubated until they reached mid-exponential phase growth. Cells of the resultant cultures were harvested and washed twice with sterile phosphate-buffered saline solution (PBS, pH 7.2) in sterile tubes at 5000 × g for 5 min.

Microorganisms were re-suspended (about 10^7 cells per mL) in culture medium RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), using two different buffers: Buffer 1 (pH 7.0 ± 0.1, 164 mM MOPS) (Sigma-Aldrich, St. Louis, MO); Buffer 2 (pH 7.0 ± 0.1, 25 mM HEPES) (Sigma-Aldrich, St. Louis, MO) 2 mM L-glutamine (Lonza, Walkersville, USA) and 2.0 g/L sodium bicarbonate (Synth, São Paulo, Brazil). The adhesion of microorganisms was performed on 24 well sterile plates (TPP Techno Plastic Products AG, Switzerland) at 37 °C in an orbital shaker (75 rpm) for 90 min (Pereira et al., 2011). The pH was measured using a benchtop pH meter (QX 1500 Plus-Qualxtron, São Paulo, Brazil).

Cell cultures were prepared with the Normal Oral Keratinocytes (NOK) (Castilho et al., 2010) and Human Keratinocyte Cell Line (HaCat) (BCRJ 0341). The cells were cultivated until they reached confluency (48 h), washed with 10 mM PBS, centrifuged and re-suspended in Dulbecco's Modified Eagle Medium (DMEM; (Gibco BRL, Grand Island, NJ, USA) and placed (about 4.5×10^4 cells/well) in 24 well plates. Live cells were counted in a Neubauer chamber (magnification ×10).

MTT assay [3-(4.5-dimethylthiazole-2-yl)2.5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) was performed at 02, 04, 06, 08 and 12 h after incubation at 37 °C in 5% CO_2 with the RPMI/MOPS, RPMI/HEPES and DMEM (control cells). After each period of contact, the culture media were removed and 250 μL of MTT/PBS solution (5.0 mg·mL^{-1}) was added to each well and incubated for 4 h at 37 °C. Next, MTT solution was removed and the formazan crystals were

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Table 1
Mean and standard deviation values of $\log_{10}$ CFU/mL for the growth of *C. albicans* and *S. aureus* in single and mixed biofilms in the RPMI/MOPS and RPMI/HEPES media, with 24 h of biofilm formation on three occasions performed in triplicate (n = 9 samples).

Group	RPMI with MOPS	RPMI with HEPES	p*
C. albicans	5.44 (±0.30)[a]	5.52 (±0.20)[a]	0.01
S. aureus	7.60 (±0.07)[b]	7.68 (±0.10)[b]	
Mixed (*C. albicans*)	6.13 (±0.25)[c]	6.13 (±0.02)[c]	
Mixed (*S. aureus*)	8.22 (±0.14)[d]	8.09 (±0.02)[d]	

* ANOVA. Tukey post-test = equal letters for results without a significant difference; the same letters for results with a significant difference.

Table 2
Mean and standard deviation values (SD) of pH of supernatant of *C. albicans* and *S. aureus* single and mixed biofilms in the RPMI/MOPS and RPMI/HEPES media on three occasions performed in triplicate (n = 9 samples).

pH of culture media			
	C. albicans	*S. aureus*	Mixed
RPMI/MOPS	6.91 (±0.02)	6.88 (±0.04)	6.89 (±0.04)
RPMI/HEPES	7.88 (±0.09)	7.53 (±0.35)	7.81 (±0.08)

solubilized in 250 μL of 2-propanol (Mosmann, 1983). The spectrophotometric measurements were performed at 562 nm (Reader 400 EZ — Biochrom, Cambridge, UK), using isopropanol as the blank.

To observe the changes in morphology, cells were analyzed and photographed under a Leica DMI 3000B inverted microscope, after 12 h of contact with the culture media.

The colony forming units (CFU/mL) and cell viability (MTT) results were statistically evaluated using ANOVA one-way followed by Tukey's test for significance $p < 0.05$. The values were expressed as the mean and standard deviation of three independent replications, in triplicate for each experimental condition (n = 9).

As RPMI supplemented with 25 mM of HEPES did not result in the growth of *S. aureus* biofilm, a further experiment was conducted where RPMI/HEPES was supplemented with L-glutamine (2.0 mM) and sodium bicarbonate (2.0 g/L). The biofilm results indicated a slight change in pH from 7.0 to a maximum of 7.8 after 24 h. However, there was no difference between RPMI buffered with MOPS and HEPES (L-glutamine/sodium bicarbonate), for the number of CFU/mL variable, showing that both buffers promoted similar biofilm growth (Table 1).

Keratinocyte cell viability was studied for up to 24 h of incubation (data not shown) with the media RPMI/HEPES pH 7.0 and RPMI/HEPES pH 8.2, resulting in a cell viability of 100% for NOK and HaCat in both conditions (Fig. 1a/b).

The cytotoxic effect of MOPS was not observed over short periods. However, the kinetic study revealed high cytotoxicity after 4 h, with similar results observed for the two cell lines (Fig. 1a/b). These results demonstrate that co-culture protocols using MOPS as buffer may not be valid, particularly for periods of incubation longer than 4 h. These

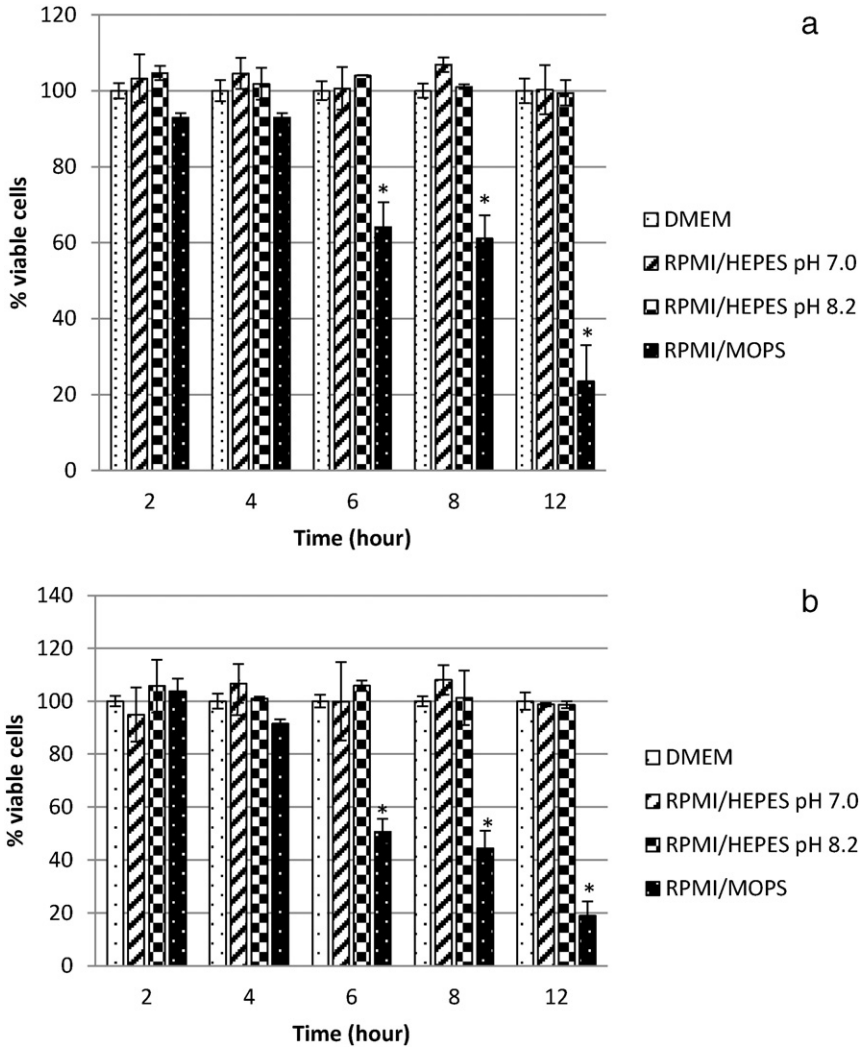

Fig. 1. (a) Viability percentage based on different culture media and pH in contact with NOK (n = 9 samples). (b) Viability percentage based on different culture media and pH in contact with HaCat (n = 9 samples). ANOVA. Tukey post-test. *significant difference.

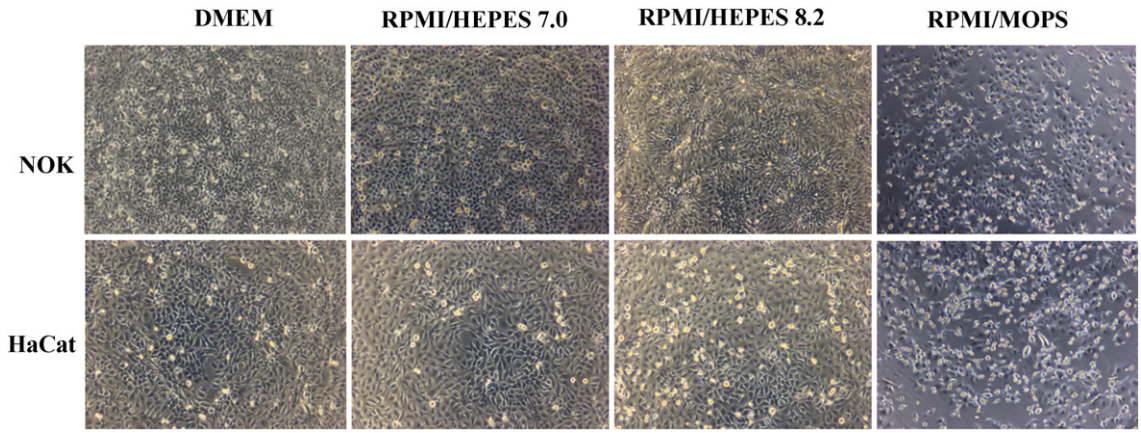

Fig. 2. Images obtained by inverted microscope for the DMEM, RPMI/HEPES pH 7.0, RPMI/HEPES pH 8.2 and RPMI/MOPS with 12 h of incubation in NOK and HaCat cell groups. Magnification ×20.

results agree with existing literature regarding the toxicity of this buffer in mammalian cells in concentrations greater than 20 mM^3. The HEPES buffer has buffering properties similar to MOPS and has been used for *S. aureus* and *C. albicans* biofilm formation (Peters et al., 2010). The medium prepared with RPMI/HEPES 25 mM, supplemented with L-glutamine (2 mM) and sodium bicarbonate (2.0 g/L) was capable of promoting both the growth of single and dual species *C. albicans* and *S. aureus* biofilms, while maintaining the cell viability of two keratinocyte cell lines around 100% until up to 12 h of incubation (Tables 1, 2 and Fig. 1b). The period of 12 h was selected to examine the morphology of the cells exposed to the different media. When cells were exposed to the RPMI/MOPS medium, a major change in viability, about 80% of cell death, was identified through MTT (Fig. 1a/b). From Fig. 2, cellular degradation in RPMI/MOPS and cell proliferation in RPMI/HEPES can be observed.

The RPMI/HEPES medium supplemented with L-glutamine (2.0 mM) and sodium bicarbonate (2.0 g/L) can be used as a viable alternative for studies willing to investigate the effects of biofilms on keratinocytes over time.

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