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Interaction of polyhexamethylene biguanide hydrochloride (PHMB) with phosphatidylcholine containing o/w emulsion and consequences for microbicidal efficacy and cytotoxicity

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ABSTRACT

Oil-in-water (o/w) emulsions containing egg yolk phosphatidylcholine (EPC) were combined with aqueous polyhexamethylene biguanide hydrochloride (PHMB). The PHMB concentration in the aqueous phase was estimated by filtration centrifugation experiments. In parallel, PHMB concentration was assessed utilizing cytotoxicity assays (neutral red) on cultured murine fibroblasts (L929 cells) and tests of bactericidal efficacy on either *Pseudomonas aeruginosa* or *Staphylococcus aureus.* Biological tests were performed in cell culture medium. Filtration centrifugation experiments demonstrated much higher aqueous PHMB concentrations than did the assays for biologically effective PHMB. Therefore, biological test systems should preferably be used to verify effective PHMB concentrations. Tests of microbicidal efficacy in which the same 0.05% PHMB o/w emulsion was re-used 8 times revealed a drug delivery system activated by the presence of test bacteria.

1. Introduction

Polyhexamethylene biguanide hydrochloride (PHMB, polihexanide) is a water-soluble polymeric cationic antibacterial agent (commercially available as Vantocil™, Cosmocil™, and Lavasept®) that has been widely used in industry and medicine. Applications include impregnation of fabrics to inhibit microbial growth [1–3], water treatment [4], contact-lens multipurpose solutions [5,6], mouthwash solution [7,8], treatment of hatching eggs to prevent *Salmonella* infection [9,10], consumer applications (swimming pool sanitizer, preservation of cosmetics), and applications in industrial processes [11]. As an antiseptic in medicine, it has been demonstrated that polihexanide is effective against fungi [12] and *Acanthamoeba* [13–15] in infective keratitis. PHMB is widely used in wound care dressings [16]. The agent differs from other known antiseptics in that it significantly promotes wound healing [17,18]. PHMB may thus become the agent of choice for the treatment of chronic wounds [19]. However, like other antiseptics, polihexanide is highly cytotoxic in the peritoneal cavity [20]. The proposed basis for the antimicrobial and cytotoxic effect is that PHMB interacts with acidic cell membrane lipids to cause phase separation and phospholipid domain formation [21].

Phosphatidylcholine is the most abundant glycerophospholipid of mammalian cell plasma membranes [22,23]. Even though it has been reported that PHMB does not react with neutral phospholipids [24], it was combined with egg phosphatidylcholine to investigate a possible protective effect on mammalian cells. The combination of an oil-in-water (o/w) emulsion containing egg yolk phosphatidylcholine (EPC) with PHMB reduces the cytotoxicity on mammalian cells without neutralizing the microbicidal efficacy against bacteria [25]. In that preliminary study [25], only selected preparations were used to determine PHMB in the aqueous phase after gentle filtration using centrifuge filter units. The present study, however, provides more details on both the interaction and the binding forces of PHMB with the EPC layer of the emulsified particles. The interaction of PHMB with the particles of the EPC-containing o/w emulsion may be described as the attachment of a compound (ligand) to a macromolecular receptor [26]. This non-covalent, reversible association of PHMB (low-molecular ligand) with macromolecular receptors (possibly phosphate groups of the EPC monolayer) can be used to derive an optimal combination of the two components suitable for use as an effective and biocompatible antiseptic preparation. The results were evaluated by assessing biologically effective PHMB using cytotoxicity assays and tests for microbicidal efficacy. Additionally, one 0.05% PHMB o/w emulsion was re-used eight times to test the antimicrobial activity based on release of the active agent from a depot in the presence of test microorganisms (a drug delivery system).

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2. Materials and methods

2.1. Chemicals

Lipoid E80 (Lipoid GmbH, Ludwigshafen, Germany) consisting of phospholipids from egg yolk was used for the preparation of the o/w emulsion.

The o/w emulsion Lipofundin® MCT 20% (B. Braun, Melsungen, Germany) containing 1.2% (w/v) EPC was manufactured by B. Braun using high pressure homogenization (30–60 MPa, 45–80 °C). The o/w emulsion was obtained from the local hospital dispensary.

Cosmocil PG (Arch Chemicals Inc., USA) containing 20% (w/v) PHMB in water was used as the antiseptic agent in this study.

2.2. Passive loading of the o/w emulsion with PHMB

Twenty percent (20%) (w/v) PHMB was diluted with sterile water (B. Braun, Germany) to final concentrations of 0.01–0.4% (w/v) PHMB. Equal volumes of the EPC-containing dispersion and 0.01–0.4% (w/v) PHMB were added to this (20 ± 2 °C) and used within 14 days after preparation.

2.3. Determination of free and EPC-associated PHMB

Aqueous and EPC-enriched lipid phases of the preparations were separated by gentle centrifugation at 380*g* (2000 rpm) at 18 °C in a Biofuge Fresco (Heraeus, Germany) using Nanosep 300 K Omega centrifugal filter units (Pall Corporation, USA) with a filter membrane of a molecular weight cutoff of 300 kDa. The centrifugal experiments with the test mixtures were repeated six times.

Concentrations of PHMB in the aqueous phase were assessed spectrophotometrically in QS 10.00 mm quartz glass cuvettes (Hellma, Germany) at 235 nm in an Ultrospec 4000 UV/VIS spectral photometer using a calibration curve of 0–20 mg/L PHMB. If necessary, aqueous filtrates were diluted with water after centrifugation to obtain sample concentrations within the calibration curve.

2.4. L929 cell line, culture medium, and cytotoxicity assays for determination of free PHMB

L929 cells (ATCC CCL1) derived from an immortalized mouse fibroblast cell line are recommended for cytotoxicity testing of medical devices in accordance with ISO 10993-5 [27]. The mouse fibroblasts (ACC 2) were purchased from the German collection of microorganisms and cell lines (DSMZ, Germany). Stock cultures of L929 cells were routinely propagated in Eagle minimum essential medium containing 10% fetal bovine serum. This culture medium with Earle's salts and L-glutamine (PAA Laboratories, Germany) was supplemented with 10% fetal bovine serum (Invitrogen, Germany) but without antibiotics to prevent additional antimicrobial activity. Double-concentrated culture medium was prepared from the powder MEM medium (Invitrogen, Germany) required for 1 L. The ingredients of the powder medium and 2.2 g $NaHCO_3$ were dissolved in 400 mL sterile water. After aseptic filtration using a 0.2-μm filter, 100 mL fetal bovine serum was added.

The neutral red assay, which was standardized for L929 cells [27], was used to assess cytotoxicity. Procedures for characterizing the resulting viability of mouse fibroblasts in the neutral red assay have been described in detail elsewhere [28–30]. Briefly, L929 cells were seeded into 96-well cell culture plates, 0.1 mL per well, at a density of 1×10^5 cells/mL. Twenty-four hours after seeding, the medium was changed and cells were cultured further for 24 h. Forty-eight hours after seeding, 0.1 mL of fresh medium (negative control) and 0.1 mL of a mixture of the same parts per volume of double-concentrated medium and PHMB-containing preparations were added. Three replicates per test sample were used. PHMB concentrations of 0–30 mg/L were selected to produce a concentration-viability curve used to assess the free PHMB concentration of the EPC-containing preparations. After 1 h of incubation in a humidified atmosphere of 5% CO_2/95% air at 37 °C, the medium was removed and the wells were washed twice for 1 min with 0.15 mL of fresh medium. 0.2 mL of a fresh sterile solution of 50 mg/L neutral red (2-amino-7-dimethylamino-2-methylphenazine hydrochloride) dye in culture medium, pre-equilibrated for 3 h at 37 °C, was then added; incubation then continued for three more hours at 37 °C. The neutral red vital dye is incorporated into the lysosomes of viable cells. After incubation, the medium was completely removed and cells were washed carefully twice with 0.25 mL of warm phosphate buffered saline (PBS) for 2 min. Incorporated neutral red dye was extracted by adding 0.2 mL of 1% (v/v) acetic acid/50% (v/v) ethanol. The plates were agitated on an orbital shaker for at least 1 h in order to ensure quantitative extraction and solubilization of neutral red. The optical densities of the wells were measured using an automated plate reader (PowerWave XS, BioTek, USA) with a 540-nm test wavelength and a 625-nm reference wavelength. The results are expressed as percentage of the viability of unexposed cells in culture medium (negative control). The separately performed neutral red assays were validated by using sodium dodecylsulfate in concentrations of 50, 100, 150, and 200 mg/L in culture medium as positive controls as recommended in ISO 10993-5 [27]. The cytotoxicity tests were repeated six times.

2.5. Microorganisms, inactivation combinations, and testing of microbicidal action

Quantitative suspension tests were applied to assess the microbicidal effect of PHMB. The trial was carried out with the test microorganisms *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442). For *P. aeruginosa,* PHMB was inactivated as recommended [31–34] by TSHC [3% (w/v) Tween 80 (63161, Sigma–Aldrich, Germany), 3% (w/v) saponin (A2542, AppliChem, Germany), 0.1% (w/v) L-histidine (1.04351, Merck, Germany), and 0.1% (w/v) L-cysteine (1.02838, Merck, Germany)]. For *S. aureus,* TSLS was used [4% (w/v) Tween 80, 3% (w/v) saponin, 0.4% (w/v) soy lecithin (A2182, AppliChem, Germany), and 1% sodium dodecylsulfate (A1502, AppliChem, Germany)]. In control experiments, TSHC inactivated PHMB without any inhibitory effect on the growth of *P. aeruginosa*, and TSLS inactivated PHMB without affecting *S. aureus* (data not shown), as recommended [35]. The microbicidal action was determined in accordance with the guidelines for testing disinfectants and antiseptics of the European Committee of Standardization [31,32].

The bacterial growth culture contained 10^8 cfu/mL in culture medium. A 0.1-ml aliquot of this inoculum was mixed with 0.9 mL of culture medium with or without (negative control) the test sample. The test sample was prepared immediately before use by combining equal parts per volume of the double-concentrated culture medium and the PHMB preparation. Aqueous PHMB concentrations of 0–30 mg/L were used to calibrate the quantitative suspension test. Incubation was performed for 1 h at room temperature. The antimicrobial agent was eliminated by transferring 0.1 mL of the incubated test combination into 0.9 mL of the inactivators TSHC (*P. aeruginosa*) or TSLS (*S. aureus*). After 5 min of inactivation, serial dilutions were prepared in inactivator, and 0.1 mL of each dilution was plated in triplicate on Trypticase Soy Agar. The cfu of the test microorganisms were counted after 48 h of incubation at 37 °C. The $\log_{10}$ reduction ($\log_{10}$ cfu/mL) was calculated according to the formula: