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Reduced cytotoxicity of polyhexamethylene biguanide hydrochloride (PHMB) by egg phosphatidylcholine while maintaining antimicrobial efficacy

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ABSTRACT

Liposomes or oil-in-water emulsions containing egg yolk phosphatidylcholine (EPC) were combined with aqueous polyhexamethylene biguanide hydrochloride (PHMB). The bactericidal activity of these preparations against *Pseudomonas aeruginosa* and *Staphylococcus aureus* as well as their cytotoxicity on cultured murine fibroblasts (L929 cells) was then assayed for either 30 min or 60 min in the presence of cell culture medium containing 10% fetal bovine serum as surrogate for wound fluid. We used two assay designs: in the first bactericidal activity and cytotoxicity were determined in separate experiments; in the second both were determined in one experiment. Combining PHMB and EPC containing o/w emulsions or liposomes protects mammalian cells without neutralizing the antiseptic effect. From all tested combinations the o/w emulsions containing 0.05% PHMB proved to be superior in this respect to the aqueous preparation.

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1. Introduction

Polyhexamethylene biguanide hydrochloride (PHMB, polihexanide) is a highly water soluble polymeric cationic antimicrobial agent which is commercially available as a 20% (w/v) aqueous solution (Vantocil™, Cosmocil™, Lavasept®). In the past, PHMB has been widely used as antimicrobial agent, i.e. in cosmetics, as sanitizer, or in contact-lens multipurpose solutions because of its low systemic toxicity. The agent accentuates from other known antiseptics due to its significant promotion of wound healing [1]. PHMB may become the agent of choice for the treatment of chronic wounds [2]. In addition to other antiseptics polihexanide is highly cytotoxic in the peritoneal cavity [3]. Therefore we proved the possibility to decrease its cytotoxicity while maintaining the antiseptic efficacy combining egg phosphatidylcholine (EPC) and PHMB.

PHMB interacts via the cationic biguanide groups with the anionic head groups in the outer layer of the cell membranes displacing membrane stabilizing Ca^{2+} [4]. The hydrophobic hexamethylene bridging domains of the polymer are relatively inflexible, so that they cannot be integrated into the hydrophobic bilayer of the cell membrane. Thus, interaction of PHMB with the cell membrane results in a bridging of the anionic head groups of adjacent acidic phospholipids [5–7]. This bridging is not restricted to pairs of adjacent acidic phospholipids but leads to a rearrangement of phospholipids into individual acidic phospholipid domains. This rearrangement results in an alteration of the biological properties of the membrane [5–9]. These alterations include repulsion of adjacent anionic phospholipids, disruption of the packaging of the phospholipid hydrophobic tails and an increase in the permeability of the membrane to low molecular weight cytoplasmic components including K^+ [10]. The ability of PHMB to create single phospholipid domains within heterogeneous phospholipid-bilayers is not restricted to microbial cell membranes. A similar increased distortion of membrane integrity is also produced in mammalian cells. The commonest glycerophospholipids in bacterial cell membranes are phosphatidylethanolamine (frequently predominant in Gram-negative species), phosphatidylglycerol and diphosphatidylglycerol (cardiolipin). The latter two are acidic phospholipids which are rarely found in mammalian cell membranes. In contrast, phosphatidylcholine (lecithin), universally present as the dominant phospholipid in membranes from animals, plants and fungi, is a very rare component of bacteria and indeed is absent altogether from Gram-positive species [11–13]. Even though it has been reported that PHMB does not react with neutral phospholipids, such as phosphatidylcholine [7], it nevertheless has considerable cytotoxicity toward mammalian cells [14–16] which contain phosphatidylcholine as the predominant glycerophospholipid.

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We therefore investigated the possibility of decreasing the cytotoxic potency of PHMB on mammalian cells while maintaining its antimicrobial efficacy by using liposomes or oil-in-water (o/w) emulsions containing almost entirely of egg yolk phosphatidylcholine forming membranous structures.

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 both the liposomes and the o/w emulsion. Lipoid E80 is composed in general of 75–84% (w/w) 3-sn-phosphatidylcholine, 7–9% (w/w) phosphatidylethanolamine, <6% (w/w) non-polar lipids, <3% (w/w) lysophosphatidylcholine, <3% (w/w) sphingomyelin, <2% (w/w) water, traces of cholesterol and D,L-a-tocopherol. 6% (w/v) lipoid E80 liposomes were prepared by high pressure homogenization by passing the dispersion in water through a Gaulin Micron Lab 40 high pressure homogenizer (APV Gaulin, Lübeck, Germany) at 70 MPa for 10 cycles. The resulting liposomes were sterile filtered using first a 0.45 μm pore size filter followed by a 0.2 μm filter. The final liposome preparation was stored under a nitrogen atmosphere.

Lipofundin® MCT 20% and Lipofundin® MCT 10% (B. Braun, Melsungen, Germany), as o/w emulsions contain 200 g/L respectively 100 g/L of 1:1 (w/w) mixture of long chain triglycerides (LCT; soybean oil) and medium chain triglycerides (MCT), glycerol (2.5%), sodium oleate (0.03%) and D,L-a-tocopherol and are stabilized with egg lecithin. These emulsions are manufactured using high pressure homogenization (30–60 MPa, 45–80 °C). The shelf life of Lipofundin® MCT 20% and Lipofundin® MCT 10% is restricted to 2 years at room temperature, which was demonstrated by continuously performed stability investigations according to a specification released by health authorities. The o/w emulsions were obtained from the local hospital dispensary.

2.2. Passive loading of liposomes and o/w emulsions with PHMB

Cosmocil PG (Arch Chemicals Inc., Norwalk, CT, US) containing 20% (w/v) polyhexamethylene biguanide hydrochloride (PHMB) in water was diluted with water to final concentrations of 0.1, 0.2, and 0.4% (w/v) PHMB. Equal volumes of EPC-containing dispersions (liposomes and o/w emulsions) and 0.1, 0.2, or 0.4% (w/v) PHMB were combined and thoroughly mixed at room temperature (20 ± 2 °C). The resulting mixtures were defined as ready-to-use preparations of PHMB and were used within 24 h after preparation.

2.3. Particle size determination of liposomes and o/w emulsion containing PHMB

The mean particle size of the preparations determined by photon correlation spectroscopy (PCS) was analyzed in a Malvern Zetasizer (Malvern Instruments, UK). PCS is a dynamic light scattering technique for determining the mean particle size of the bulk population (*z*-average) and polydispersity index (PI) as a dimensionless value for the width of particle size distribution. The PCS only can detect particles below approximately 3 μm [17]. All samples were diluted using purified water to yield a suitable scattering intensity and were analyzed at 25 °C.

2.4. 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 (Haraeus) using different centrifugal filter devices. Emulsions were centrifuged for 90 min in Ultrafree-MC filter devices (Millipore) with a microporous low-binding Durapore PVDF membrane of 0.1 μm pore size. Liposomes were centrifuged for 3 h in Nanosep 300K Omega centrifugal devices (Pall) with a filter membrane of a molecular weight cutoff of 300 kDa.

Concentrations of PHMB in the aqueous phase were assessed colorimetrically in 24-well cell culture plates at 545 nm in a PowerWave XS spectral photometer (BioTek) after reaction of a 2 mL sample with 0.1 mL 10% (w/v) sodium acetate, 0.25 mL 0.025% (w/v) Eosin Y, and 0.15 mL purified water for 15 min at room temperature in the dark [18]. A calibration curve was derived for 1–15 mg/L PHMB in water and used for calculations. 0.1 mL of aqueous filtrate of o/w emulsions or liposomes was diluted with 4.9 mL purified water to get sample solutions. 1 mL or 2 mL of the sample solution containing between 0 and 15 mg/L PHMB was used in the test.

2.5. L929 cell line, culture medium, and cytotoxicity assay

L929 cells (ATCC CCL 1), derived from an immortalized mouse fibroblast cell line, are a recommended cell line for cytotoxicity testing of medical devices in accordance with ISO 10993-5 [19]. Mouse fibroblasts (ACC 2) were purchased from the German collection of microorganisms and cell lines (DSMZ, Braunschweig, Germany). Stock cultures of L929 cells were routinely propagated in Eagle minimal essential medium (MEM) containing 10% FBS. In the cytotoxicity assay cells were grown in medium containing 10% fetal bovine serum (FBS), which is similar to the composition of artificial wound fluid [20].

MEM with Earle's salts and L-glutamine (PAA Laboratories, Cölbe, Germany) was supplemented with 10% FBS (Invitrogen, Karlsruhe, Germany). Double concentrated culture medium (2×MEM) was prepared from powder MEM medium (Invitrogen, Karlsruhe, Germany) for 1 L. The ingredients of the powder medium and 2.2 g $NaHCO_3$ were dissolved in 400 mL water. After aseptic filtration using a 0.2 μm filter 100 mL FBS was added. The cell culture media were used without antibiotics to avoid possible interactions. The neutral red (NR) assay has been standardized for L929 cells [19]. Procedures for the NR assay have been described in detail elsewhere [21–23]. Briefly, L929 cells were seeded into 96-well cell culture plates, 0.1 mL/well, at a density of 1×10^6 cells/mL to reach about >90% confluence after 24 h. 24 h after seeding, 0.1 mL fresh medium and mixtures of the same parts per volume of 2×MEM and ready-to-use preparations of PHMB (see Section 2.2) were added. Six replicates per test sample were used. After 30 min or 60 min 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 1–2 min with 0.15 mL of fresh medium. 0.2 mL of a fresh sterile solution of 50 mg/L NR (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) dye in medium, pre-equilibrated for 3 h at 37 °C was then added for 3 h at 37 °C. The NR vital dye is incorporated into the lysosomes of viable cells. After incubation, the medium was completely removed and cells were washed carefully twice with of 0.25 mL of warm PBS for 2 min. Incorporated NR 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 solubilisation of NR. The optical densities of the wells were measured using an automated plate reader (Bio-Rad, Benchmark) with a 540 nm test wavelength and a 655 nm reference wavelength. The results are expressed as percentage of the control (unexposed cells). The cytotoxicity tests were repeated three times.

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