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Development of a slow-release device for pocket disinfection

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Abstract

Polyhexanide (polyhexamethylene biguanide) (PHMB) is a polymeric substance that is broadly used as disinfectant and antiseptic. It was shown to be suitable for clinical use in critically colonized or infected acute and chronic wounds since it exhibits a broad antimicrobial spectrum combined with good cell and tissue tolerability and low risk of contact sensitization. Furthermore a wound healing promoting effect was reported. The aim of this work was to develop a PHMB containing slow release device for sustainable disinfection of bacterial infected periodontal pockets and other colonized niches of the body.

A preparation of 10% PHMB in paraffin microcapsules with the size of 200 µm was developed. Its antimicrobial and releasing properties were investigated *in vitro* using dissolving experiments in hanks buffered salt solution (HBSS) with and without fetal calf serum (FCS), development of *Streptococcus mitis* (*S. mitis*) growth inhibition zones on blood agar plates and mass spectrometry.

The microcapsules dissolved constantly over a time period of 32 days. The supernatants from the dissolving microcapsules that were used for the growing experiments of *S. mitis* on agar plates, demonstrated a time dependent development of inhibition zones of *S. mitis* growth from 1948 µm (24 hours) up to 633 µm (32 days) during the whole time of experiments that were still present after 32 days. Mass spectrometry of the supernatants revealed, that PHMB was detectable after every time point up to 32 days in concentration range from 4027 ng/µl (24 hours) to 930 ng/µl (32 days).

In conclusion, an anti-infective device with low toxicity, good tissue tolerance and high antibacterial efficacy which can be used as a long-term therapeutic agent with slow-release functionality was established.

Keywords: Polihexanide; Periodontitis; Periodontal pockets; Slow release device; Antibacterial; Disinfection

1. Introduction

Polyhexanide (polyhexamethylene biguanide, PHMB) is a polymeric substance broadly used as disinfectant and antiseptic suitable for clinical use in critically colonized or infected acute and chronic wounds. Its beneficial characteristics are particularly attributable to its broad antimicrobial spectrum, good cell and tissue tolerability, ability to bind to the organic matrix, low risk of contact sensitization, and wound healing promoting effect [1]. In addition, no development of microbial resistance during PBMB use has been detected to date, nor does this risk appear imminent. The aim of therapy using PBMB is to reduce the pathogen burden in a critically colonized or infected acute or chronic wound [1].

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PHMB is a Lewis base and interacts with on acidic Lewis part of phospholipids in the bacterial membrane and binds to the positively charged part of it. That leads to increased fluidity, permeability and loss of integrity, followed by the death of the organism [2-5]. Due to its nonspecific, strong interaction with negatively charged phospholipids, PHMB has a broad antimicrobial spectrum, including Gram-positive and Gram-negative bacteria, plaque-forming and biofilm-building bacteria, spore-forming bacteria (but not bacterial spores), and intracellular bacteria such as *Staphylococcus aureus, Bacillus subtilis*, *Streptococcus faecalis*, *Streptococcus lactis*, *Escherichia coli, Enterobacter cloacae*, *Pseudomonas aeruginosa, Saccharomyces cerevisiae,* chlamydiae and mycoplasma, and fungi including *Candida* spp. as well as *Aspergillus* spp. [6-9]. PHMB is classified as 'practically nontoxic', based on the low oral toxicity of 5 g/kg in rat [10]. PHMB showed neither sensitizing nor photosensitizing effects in animal tests. In contrast to chlorhexidine that is regularly reported to lead to late-onset hypersensitivity, eczema and even to severe anaphylactic reactions, PHMB seems to carry only a slight allergic risk and remains an uncommon contact allergen [10-14]. *In vitro* results from cell culture tests and explant tests show, that PHMB has remarkably low cytotoxicity as shown in murine fibroblasts and rat heart tissue and fetal rat humeri as well as tests on wound healing in a guinea pig model [15,16].

In conclusion the outstanding relation of PBMB between antimicrobial efficacy and low cytotoxicity and its exceptional tissue compatibility makes it a promising anti-infective substance with highly interesting features.

Chlorhexidine (CHX) is a long known anti-septic substance that is routinely used for the treatment of gingivitis and periodontitis. [17,18]. It has been considered as gold standard in chemical plaque-control and periodontal anti-infective therapy [19-21]. Referring to the manufacturer PerioChip® is a biodegradable chip, which contains 2.5 mg of chlorhexidine gluconate. It is a thin wafer that is inserted under the gums in those areas where pockets are 5 mm or deeper. Because of the biodegradable gelatin matrix it dissolves with no need of removal. It has been shown, *in vitro*, to fight bacteria for a period of seven to ten days. In a randomized clinical and microbiological trial including 15 patients diagnosed with chronic periodontitis analyzing the major periodontopathogenic bacteria *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* a significant improvement in all clinical variables in the test group as compared to the control group was demonstrated. Total colony counts were significantly reduced in the test group as compared to control over the study period [22].

CHX is increasingly used not only as an antiseptic to prevent hospital infections and an adjuvant in oral hygiene but also as a preservative in personal care products. Following more widespread exposure to the agent, reports of adverse reactions are increasing. Complications can range from mild irritant contact dermatitis to life-threatening anaphylaxis. In some cases allergic contact dermatitis preceded anaphylaxis [23].

Hydrolyzed gelatin is of animal origin (porcine, bovine) and besides the risk of allergic reactions against foreign proteins gelatin is not suitable for a vegetarian alimentation and because of the porcine fraction it may be refused by persons of muslim belief.

Aim

The aim of this study was to investigate the properties of newly developed PHMB containing test samples prepared of different materials. The kinetics of the active component release was analyzed as well as the anti-bacterial efficacy.

2. Material and methods

Polyhexanide (PHMB) containing micro particles with a PHMB content of 10% and 54% paraffin as carrier material were used. The particle size was 200 µm ± 50 µm

2.1. Analysis of the release kinetics of the PHMB samples

For the investigation of the release kinetics, the particles were suspended in 5000 µl HBSS with 10 mM HEPES buffer and with or without 50% fetal calf serum (FCS) in a PHMB concentration of 10 µg/µl. The substances were incubated at 37°C under constant stirring. After 24 h hours (h), 2 days (d), 4d, 8d, 16d and three weeks the samples were investigated visually and the grade of resolution was controlled and recorded.

A sample of 1000 µl particle free supernatant was harvested, the last one after complete resolution, for analysis of the antibacterial properties. The volume removed was replaced after every sampling. As negative control HBSS with or without FCS was used.

All samples were prepared in duplicate in 3 different experiments.

2.2. Analysis of the anti-bacterial properties of the PHMB samples

The blood agar plates were coated with *Streptococcus mitis (S. mitis)* cultured in BHI, 100 µl bacterial solution was distributed over the plates using a microbiological bail. Gaps were punched out from the agar plates using a biopsy punch.

The PHMB containing solutions and the negative control from every incubation time (40 µl) were placed in the gaps. The plates were incubated for 24h at 37 °C.

After the incubation time, the plates were visually analyzed respective to development of zones of bacterial growth inhibition. The inhibition zones were measured and photographed in a microscope with integrated unit of length measurement and camera.

2.3. Statistical analysis

The results were analyzed using independent two-sample Student´ s test. The character of the evaluation was explorative. The probability of error was set at 5% and is shown as *p*-values.

2.4. Quantification by mass spectrometry

Chromatographic conditions: VDSper Pur 100 SL columns were used with a particle size of 4µm and a length × inner diameter of 150 x 2.0 mm. The separation mode was analytical using normal phase. The solvent A of the mobile phase was 15 mM ammonium formiate with pH 3.5 and solvent B was acetonitrile. The elution conditions were the following: 0 min 10% solvent A, 1 min 10% solvent A, 10 min 80 solvent A, 12 min 80 % solvent A, 14 min 10% solvent A and 25 min 10% solvent A. The flow rate was 300 µl/min. Injected were 20 µl, the column temperature was 30^0 C with a pressure of 70-160 bar. The HPLC system was the Alliance HT Waters 2790 with waters micrpmass ZQ 4000, the detection was done by +ESI mass trace with the PHMB (dimer) m/z 367.

3. Results

3.1. Antibacterial properties

The mean inhibition zone of the growth of *S. mitis* using paraffin particles (Fig. 1) containing 10% PHMB in HBSS with 50% FCS developed from 1342 µm after 24h (A) to 1512 µm, 1425 µm, 1262 µm, 825 µm, 665 µm to 633 µm after 2 days (B) 4d (C), 8d (D), 16d (E), 28d (F) and 32d (G) ; a graphics with the kinetics of the inhibition zone development is shown in Fig. 1H.

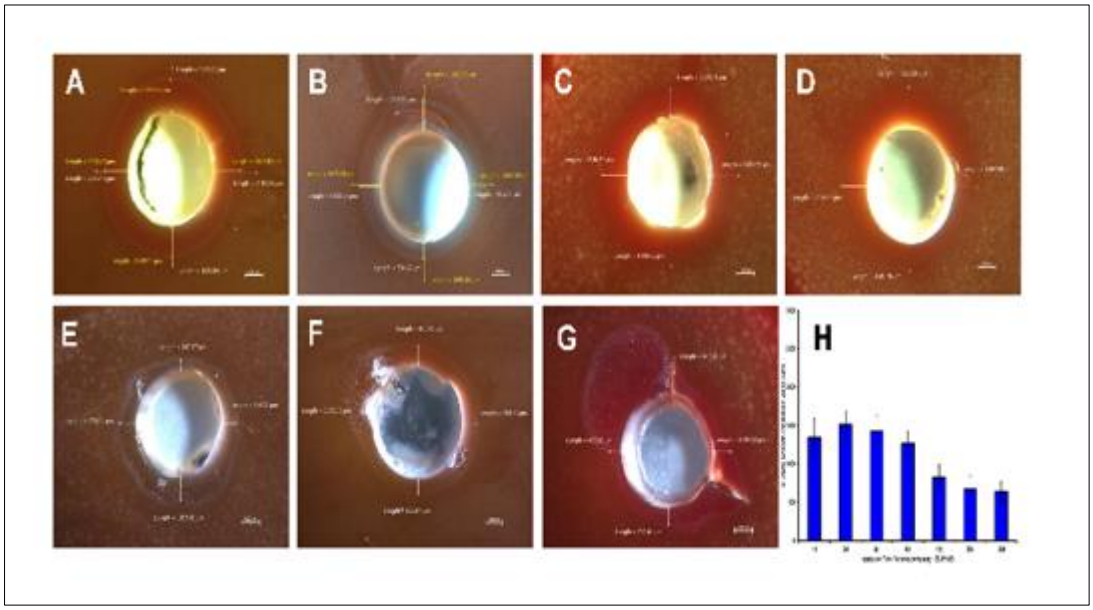

Figure 1 The mean inhibition zone of the growth of S. mitis using paraffin particles containing 10% PHMB in HBSS with 50% FCS after 24 hours (h) (A), 2 days (d) (B), 4d (C), 8d (D), 16d (E), 28d (F) and 32d (G). Fig. 1H shows a graphics of the kinetics of the inhibition zone development

The mean inhibition using HBSS without serum (Fig. 2) altered from 1948 µm after 24h (A) to 1678 µm, 1299 µm, 1235 µm, 1259 µm, 1235 µm and 1128 µm after 2d (B), 4d (C), 8d (D), 16d (E), 28d (F) and 32d (G) ; a graphics with the kinetics of the inhibition zone development is shown in Fig. 2H.

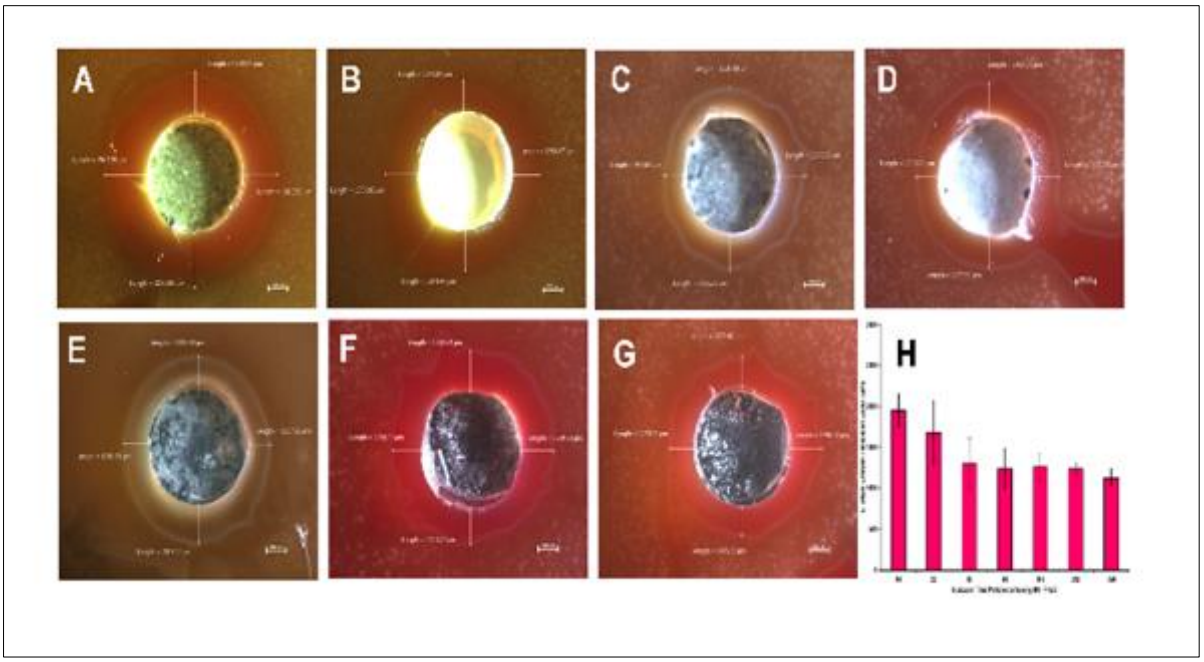

Figure 2 The mean inhibition zone of the growth of *S. mitis* using paraffin particles containing 10% PHMB in HBSS without FCS after 24 hours (h) (A), 2 days (d) (B), 4d (C), 8ds (D), 16 d (E), 28d (F) and 32d (G). Fig. 2H shows a graphics of the kinetics of the inhibition zone development

In both cases the paraffin particles were partly melted already after 24h and the solution appeared increasingly turbid and milky. Fig. 3 shows the development of the inhibition zones with/without serum in comparison over the time.

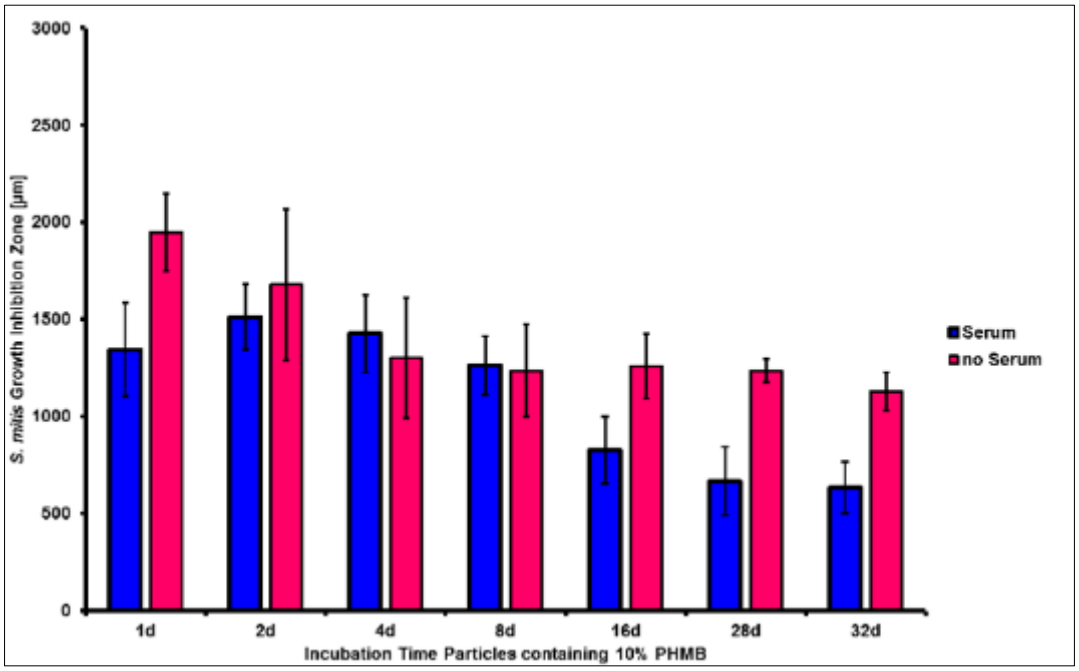

Figure 3 Development of the inhibition zones with/without serum in comparison over the time from 24h to 32d

3.2. Release kinetics by quantification using mass spectrometry

Samples without serum from the *in vitro* experiment (Fig. 4):

After 24h in 2µl sample volume 4027 ng (2014 ng/µl) PHMB were detected, 3456 ng (1728 ng/µl) after 48h, 3161 ng (1581 ng/µl) after 4d, 2961 ng (1481 ng/µl) after 8d, 2538 (1269 ng/µl) after 16d, 1970 ng (985 ng/µl) after 28d and 1806 ng (903 ng/µl) after 32d.

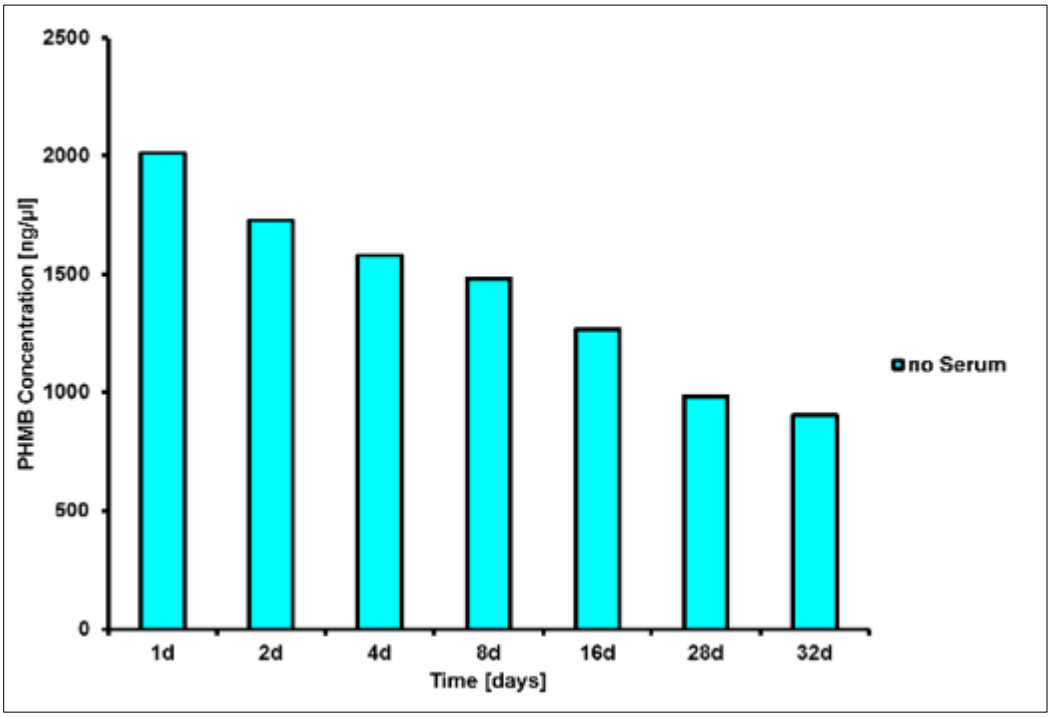

Figure 4 Release kinetics by quantification using mass spectrometry from samples without serum over the time from 24h to 32d

Samples with 50% serum (FCS) from the *in vitro* experiment (Fig. 5):

In comparison to the samples without serum after 24h in 2µl sample volume 60.03 % of the PHMB concentration was detected, 72.93 % after 48h, 53.86 % after 4d, 52.73 % after 8d, 34.92 % after 16d, 40.1 % after 28d and 34.42 % after 32d.

3.3. Stability of the antibacterial efficacy

The antibacterial efficacy of PHMB remained detectable over the whole time period of 32d.The particles were partly melted after 24h stirring in 37°C and an emulsion developed. The PHMB was released from the beads in the following kinetics: After the first 24h 1/5 of the calculated PHMB concentration (10 µg/µl) was detected (4.027 µg in 2 µl). The concentrations that were quantified in the samples from 48h, 4d, 8d,16d, 28d and 32d were 86%, 79%, 74%, 63%, 49% and 45% of the 24h value (3.456 µg, 3.161 µg, 2.961 µg, 2.538 µg, 1.970 µg and 1.806 µg in 2 µl respectively). The presence of serum seemed to inhibit the release or the detection of the PHMB.

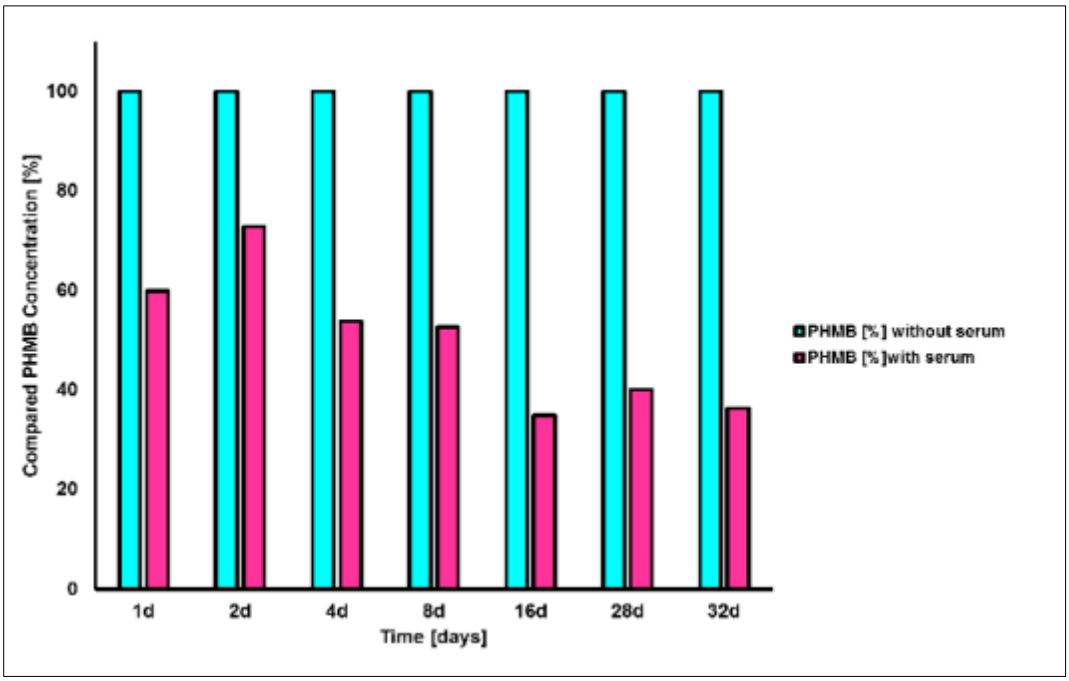

Figure 5 Release kinetics by quantification using mass spectrometry from samples with 50% serum in comparison to the samples without serum over the time from 24h to 32d

4. Discussion

Therapy of acute or chronic inflammations is trying to stop the pathogenic processes causing the illness and to support the healing processes by removal of the pathogens. In this respect in the past so-called "slow-release" devices have been developed, which release pharmaceutically active compounds/substances. In periodontics locally applied antimicrobials should demonstrate a slow release of active components, because the constant flow of exudate out of the periodontal pocket is causing a dilution of the therapeutic products in the pocket [24].

In a study that retrospectively evaluated the clinical outcomes of subgingival debridement (e.g. scaling and root planing, SRP) and application of either Arestin (AR) minocycline microspheres or a chlorhexidine chip (PerioChip, PC) in patients with chronic periodontitis during supportive periodontal treatment (SPT) it was found that both treatments led to a reduction in pocket depth (PD) and gain of clinical attachment levels (CAL). AR showed higher improvements in pockets of ≥7 mm compared with PC while PC showed more effect in 5-6 mm PD [25]. A further study assessed the *in vitro* antimicrobial activity of biodegradable polymer formulations containing a new minocycline lipid complex (P-MLC) in comparison to a pure minocycline or an existing commercial formulation showing activity against a six-species composed periodontal biofilm. As result, the biofilm development was demonstrated to be clearly inhibited by all tested formulations containing minocycline without clear differences between them while eluates of experimental formulations showed improved antimicrobial activity. Eluates of one experimental formulation (P503-MLC) was able to inhibit biofilm formation at 28 days, with a diminution by 1.87 $\log^{10}$ colony forming units (CFU) in comparison to the untreated control. The new experimental formulations were easy to insert in periodontal pockets and so the authors concluded that they could display alternatives in local antimicrobials, and that this would be worth for further testing [26].

Here a gelatin-free alternative is described, using PHMB as active ingredient instead of CHX and exerting a much longer time-span during which the active ingredient (PHMB) is released in therapeutic concentrations. The field of application of the PHMB-granula is primarily the area of wound-disinfection and the therapy or prevention of acute or chronic inflammations, especially the disinfection of niches and not only periodontal pockets in the area of dental and medical treatments. Possible other areas of medical treatment where niche-disinfection or pocket-disinfection is required, e.g. regarding complications with diabetic necrosis and other illnesses, the PHMB-granula can be applied also successfully. It is applicable for humans and/or animals.

Infection i.e. with high-risk human papilloma viruses (HPVs) is associated with cervical lesions. Treatments for these lesions induced by HPV infection include surgical, topical, immunomodulatory or destructive therapy. Post recovery

therapy mostly involve analgesic, topical, anti-inflammatory and antimicrobial treatments to reduce the risk of local infections. Especially, CHX-based products are used as vaginal antiseptic over three decades and, some time later, the development of biguanide-derivatives allowed to identify more effective and less toxic substances, including PHMB, that has been widely used as an alternative for antibiotics in a variety of local anti-infective therapy. Despite the *in vitro* proven better tissue compatibility and antimicrobial activity of PHMB compared to CHX, clinical reports comparing the two antiseptics are rare. A study investigated the efficacy and safety of PHMB-based vaginal suppositories in comparison to a similar chlorhexidine-based treatment, in the post recovery regimen after surgical treatment of cervical lesions. As result, PHMB-based treatment demonstrated enhanced efficacy compared to chlorhexidine, regarding the healing process and prevention of bacterial infections. The authors concluded, that, because of its safe and effective properties, the vaginal treatment with PHMB is beneficial compared to CHX. These results are in accordance with previously reported *in vitro* evidences [27].

In contrast to the disinfection particles already known, which provide release of CHX for only about 2 weeks, the microcapsules demonstrated in this study a slow release of PHMB for at least about twice as long, provide PHMB instead of CHX and do not contain animal material (gelatin). The antibacterial efficacy of PHMB stayed detectable over the whole time-period of observation (32d). While not wanted to be limited by theory, the particles probably melt after 24h stirring in 37°C and form an emulsion, whereby the process of melting supports/enhances the release of the at least one pharmaceutical active substance contained within them.

5. Conclusion

In conclusion, the results of this study provide the base for the development of an anti-infective device with low toxicity, good tissue tolerance and high antibacterial effectivity that can be used as long-term therapeutic agent with slow-release functionality. This device can be beneficial not only for infected periodontal pockets but for all bacterial colonized niches of the body.

In the year 2013 according to the hazardous substances law following the European Chemicals Act PHMB was classified as category 2 “may presumably cause cancer”.

Products that contain more than 1% PHMB since then had to be indicated as category 2.

For risk assessment two criteria are crucial, the dermal resorption and the relevance of the postulated carcinogenesis. Up to 2014 there was no evidence for resorption of PHMB after usage on skin and wounds [1]. Toner (2014) performed a methodological rarely relevant *in vitro* study with in advance frozen human skin to investigate the resorption of PHMB resulting in the conclusion that PHMB persists in the stratum corneum [28]. Nevertheless the SCCS uses this study to suggest the resorption of PHMB by the skin. Unfortunately the Scientific Committee on Consumer Safety (SCCS) interpreted this study erroneous [29]. Toner (2014) performed 20 strippings of the frozen split skin – doing so he stays in clearly the stratum corneum since stripping can be performed up to 100 x until the stratum corneum is removed. The SCCS provides the following false interpretation: “The SCCS notes that 20 tape strippings were used to remove stratum corneum, which is a high number. Normally, not more than 5 tape strippings should be used to remove stratum corneum. Thus, it cannot be excluded that some absorbable amounts of PHMB were removed by the high number of tape strippings used.” This clearly is not correct, since, as mentioned before, it needs up to 100 strippings until the removal of the stratum corneum. For the acute toxicity of PHMB the classification “practically non-toxic” arises and there are no adsorptive-toxic effects, including mutagenicity und teratogenicity, known. A toxic effect, like expected, is detectable only after usage of concentrations that are markedly higher than the maximal tolerable dose and for the practice of no relevance [30].

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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