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Antimicrobial activity of zinc oxide particles on five micro-organisms of the Challenge Tests related to their physicochemical properties

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ABSTRACT

Zinc oxide is commonly used in pharmaceutical products to prevent or treat topical or systemic diseases owing to its antimicrobial properties, but it is scarcely used as preservative in topical formulations. The aim of this work was to investigate the antimicrobial activity of zinc oxide (ZnO) powders on the five microbial strains used for Challenge Tests in order to evaluate this inorganic compound as a preservative in topical formulation and assess relationships between the structural parameters of ZnO particles and their antimicrobial activity. For this purpose, the physicochemical characteristics of three ZnO grades were measured and their antimicrobial efficacy against the following micro-organisms – *Escherichia coli*; *Staphylococcus aureus*; *Pseudomonas aeruginosa*; *Candida albicans*; *Aspergillus brasiliensis* – was assessed using disc diffusion susceptibility tests and a broth dilution method. The comprehensive dataset of physicochemical characteristics and antimicrobial activities (MIC and MBC) is discussed regarding methodological issues related to the particulate nature of ZnO and structure–activity relationships. Every ZnO grade showed bactericidal and antifungal activity against the five tested micro-organisms in a concentration dependent manner. ZnO particles with smaller size, larger specific area and higher porosity exhibit higher antimicrobial activity. Such trends are related to their mechanisms of antimicrobial activity.

1. Introduction

Zinc oxide (ZnO) shows attractive antimicrobial properties that are utilized in several pharmaceutical and cosmetic products. Zinc is also an endogenous metal that is involved in many physiological phenomena. Zinc is involved *in vivo* in more than 300 enzymatic reactions as a cofactor (Haase et al., 2008; Tapiero and Tew, 2003; Vallee and Falchuk, 1993). In medications for topical use, ZnO acts as a soothing and protective coating against skin irritation and abrasions, as a mild astringent, and as an antimicrobial agent. It is commonly used to treat diaper rash, acne, and minor burns (Arad et al., 1999). ZnO regulates skin overactive sebaceous glands functions and dries up excess sebum. It is also extensively used as protective agent in sunscreen products because of its ability to filter UV radiations (Anthony et al., 1987; Pinnell et al., 2000).

Though its antimicrobial activity is well-established, a survey of the actual pharmaceutical and cosmetic products and of the literature reports did not reveal so many utilizations of ZnO in pharmaceuticals, either as active pharmaceutical ingredient or as preservative. Microbial spoilage of topical formulations has always been of special concern for pharmaceutical and cosmetic industries where preservatives taken in a list of authorized compounds prevent contamination by micro-organisms during storage and utilization. However, health industries are facing new restrictions regarding the use of preservatives since few years. Paraben preservatives started being questioned after new studies have been published by Darbre et al. (2002, 2003, 2004), who reported links between the daily exposure of paraben preservatives and both breast cancer and endocrine-disruption issues. Among alternative materials exhibiting antimicrobial properties, ZnO appears promising because of its high antimicrobial activity (Sawai, 2003; Sawai and Yoshikawa, 2004), low toxicity, and easy clearance. Since the report by Favet et al. (2001) on the antimicrobial properties of ZnO in the ointment zinc gelatin described in the Swiss Pharmacopoeia, only few formulations containing ZnO as an antimicrobial agent have been reported so far (Jones et al., 2008; Zhang et al., 2008; Tayel et al., 2011; Woost et al., 2012).

Specific studies devoted to the antimicrobial efficacy of ZnO in the context of topical product preservation are sparse and appeared somehow contradictory. The several reports dealing with

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the antimicrobial activity of ZnO, the relevant structure–activity relationships, and the fundamental mechanisms relied on investigations that took into account the influence of one single characteristic of ZnO and few microbial strains only. Such studies carried out on different microbial strains and types of ZnO particles could not reach a definite view regarding the mechanisms and the parameters that control the antimicrobial activity because of the large scatter of microbial strains and experimental methods used in such studies. As example, (Sawai, 2003) showed that antibacterial activity of ZnO was stronger against Gram-positive (*Staphylococcus aureus*) than Gram-negative bacteria (*Escherichia coli*), which was confirmed by other authors (Amornpitoksuk et al., 2011; Emami-Karvani and Chehrazi, 2011; Li et al., 2009; Reddy et al., 2007), but was contradicted by Applerot et al. (2009, 2010). Several studies have shown that the concentration, size and surface area of ZnO particles influenced their antimicrobial activity (Yamamoto, 2001; Fang et al., 2006; Applerot et al., 2009; Jiang et al., 2009; Tayel et al., 2011). At variance with previous claims, Amornpitoksuk et al. (2011) reported that the antimicrobial activity of ZnO did not depend on the particle size and shape. At the end, it is difficult to figure out the origin of such conflicting results because the types of ZnO particles, the studied microbial strains and the experimental protocols were not the same. Such issues call for more consistent investigations that take into account several microbial strains and various types of ZnO particles at different concentrations by using the same experimental protocols.

The aim of the present work is the comparison of the sensitivity of several microbial strains to ZnO and an evaluation of the physicochemical characteristics of ZnO responsible for its antimicrobial activity. Three grades of ZnO particles having different physicochemical characteristics have been evaluated for their antimicrobial activity against the five micro-organisms used in the Challenge Tests. The antimicrobial was first estimated on solid agar plates by means of a disc diffusion susceptibility test. A quantitative assessment of the activity was done in a second stage by means of a liquid broth dilution test. Methodology of microbiological tests was adapted to powder materials. The systematic study was aimed at (i) determining the concentrations at which ZnO exhibited an antimicrobial activity against these five microorganisms, (ii) searching for the physicochemical properties of ZnO that influenced the antimicrobial activity.

2. Materials and methods

2.1. Materials

Various ZnO powders of pharmaceutical grade were studied: ZnO-1 from Rockwood Pigments (Beltsville, Maryland, US); ZnO-2 from SILOX (Engis, Belgium); ZnO-3 from Zinc Corporation of America (Pittsburgh, Pennsylvania, US). Synthesis protocols were not given by the suppliers. The microbiological quality of each powder was evaluated before microbiological tests in order to be sure that these raw materials were not contaminated. The ZnO samples available as dry powders were dispersed in liquid media (water or Mueller-Hinton broth) by means of ultrasounds. Vials containing ZnO and the liquid medium were placed in an ultrasonic cleaning bath Elmasonic S 30H (Elma GmbH, Germany) operating at full power (90 W) for 1 h.

2.2. Physicochemical characterization of ZnO particles

Transmission Electron Microscopy (TEM) was performed at the 'Centre Technologique des Microstructures' facility (University of Lyon) on a Philips CM120 microscope operating at 80 kV acceleration. A dilute aqueous suspension (0.1%) was spread on Formvar/carbon grids and dried before observation.

X-ray diffraction analyses were performed on dry powders at the 'Centre Henri Longchambon' facility (University of Lyon) using a Bruker AXS D8 ADVANCE X-ray diffractometer operating with the Cu $K\alpha 1$ line at 1.54 Å wavelength.

The specific surface area and the porosity of ZnO samples were determined by nitrogen adsorption measurements using a Tristar 3000 Micromeritics BET instrument. The specific area was determined by the Brunauer–Emmett–Teller (BET) multipoint method and the pore volume was analyzed by the Barrett–Joyner–Halenda (BJH) method.

Particle size distributions of ZnO suspensions in water and in nutrient broth were measured by low-angle laser light scattering using a Malvern Mastersizer 2000 instrument. Particle size distributions were calculated according to Mie theory using the refractive indices of water (1.33), MH broth (1.33647) and ZnO (2.008). The median diameter $D(0.5)$ was retained as a characteristic parameter.

2.3. Antimicrobial tests

2.3.1. Microbial strains and culture conditions

Microbial strains were purchased from the culture collection of the Institut Pasteur (France): *E. coli* CIP® 53.126 (equivalent strain ATCC® 8739); *S. aureus* CIP® 4.83 (ATCC® 6538); *Pseudomonas aeruginosa* CIP® 82.118 (ATCC® 9027); *Candida albicans* IP 48.72 (ATCC® 10231); *Aspergillus brasiliensis* IP 1431.83 (previously named as *Aspergillus niger;* ATCC® 16404). They were stored in cryovials at −20 °C.

Bacterial stock cultures were obtained by inoculation of a Trypcase Soy Agar (TSA) plate (BioMérieux, France) with a single bead from a cryovial. After incubation at 32.5 °C overnight, a colony was pricked out to prepare the bacterial working culture on a TSA plate incubated at 32.5 °C during 24 h. Fungal stock cultures were prepared by inoculation of a Sabouraud Gentamicin Chloramphenicol 2 (SGC2) plate (BioMérieux, France) with a bead, followed by incubation at 22.5 °C during 48 h for *C. albicans* and five days for *A. brasiliensis*. The fungal working cultures were prepared by pricking out a colony on a SGC2 plate incubated at 22.5 °C during 48 h for *C. albicans*.

Microbial suspensions were prepared in a tryptone-salt broth (BioMérieux, France). The turbidity of bacterial suspensions was adjusted to 1.5×10^8 CFU mL^{-1} by comparison with the 0.5 McFarland Standard (BioMérieux, France). The *C. albicans* yeast suspension was prepared at a concentration of 4.0×10^7 CFU mL^{-1} (McFarland 4.0 standard). The *A. brasiliensis* mold suspension was prepared and its concentration was calibrated by counting the number of spores on a Malassez cell to reach 1.5×10^7 spores mL^{-1}. For each experiment, the real microbial concentration was confirmed by plate counts.

2.3.2. Disc diffusion susceptibility tests

The disc diffusion assay was carried out by swabbing each strain on Mueller-Hinton (MH2) solid agar plate (BioMérieux, France) using the 1/10 dilution of the microbial suspensions. Sterile standard discs of cellulose (6 mm diameter, BioMérieux, France) were impregnated with sterile aqueous suspensions of ZnO at 1%, 5% and 10% w/w concentrations and placed onto the agar surface. A disc impregnated with only sterile water was taken as a negative control. After overnight incubation in a thermostatic chamber in the dark at 32.5 °C for the bacterial strains and 48 h at 22.5 °C for the fungal strains, zones of inhibition around each disc were observed.

2.3.3. Broth dilution tests

Quantitative tests in liquid media were performed using freshly prepared ZnO suspensions in Mueller-Hinton (MH) broth (AES