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Bacterial Endotoxin Released by Different Types of Mouthwash

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Abstract: Endotoxins are part of the outer membrane of the cell wall of gram-negative bacteria. Mouthwashes or rinses are products used to enhance oral hygiene. In the present study, gram positive and gram negative bacterial isolates were collected and laboratory tests was used to assess the inhibition of growth of oral bacteria by three modern commercially available mouthwashes containing Aloe vera, Peppermint and Orasept. The action of each mouthwash was studied using electron microscopic studies and end-point Microtitre plate. The *in vitro* liberation of endotoxin was found to be induced with selected types of mouthwash targeted toward, bacterial endotoxin. Specific mouthwash have been classified here as "weak" (peppermint), because a high concentrations is needed to kill or inhibit the growth of bacteria, or "strong" (e.g. Orasept) where only a low concentration of mouthwash is required.

Key words: Endotoxin · Mouthwash · Toxicity · Lipopolysaccharides (LPS) · End-point method

INTRODUCTION

Mouthwashes are solutions or liquids which are used to rinse the mouth and which generally contain antiseptic, astringent and breath-sweetening agents which are employed to clean the mouth and teeth. These topical antimicrobial should be categorized into one of two categories or generations based on their pharmacological properties, namely that such agents can kill bacteria on contact or exhibit a direct antibacterial effect [1-5].

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria and are invariably associated with Gram-negative bacteria whether the organisms are pathogenic or not [6]. Although the term "endotoxin" is occasionally used to refer to any cell associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide (LPS) complex associated with the outer membrane of Gram-negative pathogens such as *Escherichia coli*, *Bordetella pertussis*, *Haemophilus influenzae*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria* and *Vibrio cholerae*.

Antibiotics may inhibit bacterial growth or may kill bacteria by inhibiting cell wall synthesis or protein synthesis. The amount of endotoxin released during antibiotic action has been found to be clinically important [7].

Mouthwash or mouth rinses are used to enhance oral hygiene. Anti-cavity mouth rinse uses fluoride to protect against tooth decay [8]. It is, however, generally agreed that the use of mouthwash does not eliminate the need for both brushing and flossing. The American Dental Association suggest that regular brushing and proper flossing is sufficient, in most cases, to maintain oral hygiene and that mouthwashes should only be used short term. Exposure to LPS, or dusts containing bacterial endotoxin should be taken seriously as a potential problem and should be taken into account during health examinations [9-15]. The aim of the present study was to determine the ability of a range of mouthwashes to induce the release of bacterial endotoxin.

MATERIALS AND METHODS

Collection of Bacterial Isolates and Mouthwashes: *Staphylococcus aureus* (G^+), *Staphylococcus epidermidis* (G^+), *Bacillus subtilis* (G^+) and Gram negative bacterial isolates such as *Salmonella* sp (G-), *Pseudomonas aeruginosa* (G-), *Serratia* sp (G-), *Klebsiella pneumonia* (G-), *Enterobacter* sp (G-) *Escherichia coli* (G-) and *Proteus* sp (G-) (obtained from King Saud Medical Complex (KSMC) and King Khalid University Hospital (KKUH) Riyadh, Kingdom of Saudi Arabia) and

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maintained in Nutrient agar medium. Three kinds of mouthwash products were used, namely: Aloe vera (Al), Peppermint (Pe) and Orasept (Or).

Electron Microscopic Studies: The inoculum was grown in LB broth media at 37°C for 18 to 24 hours to obtain an optical density of 1.3 at 420 nm equivalent to 10^8 C.F.U ml^{-1}. The cells were collected by centrifugation at 3000 xg for 5-15 min. Supernatants were discarded and the bacterial pellet were washed in PBS (pH 7). Suspensions of each previous inoculum (1ml) in PBS (containing 10^8 of C.F.U ml^{-1}) were then centrifuged at 3000 xg for 5-15 min. The supernatant was then discarded and the bacterial cells were treated with 1ml of PBS and the MIC of the mouthwash products (Al, Pe and Or). Incubation was at 37° C for 24 hours with shaking (100 rpm).

The suspensions were then centrifuged at 3000 xg for 5-15 min and supernatants were discarded. The bacterial cells were gently washed twice with PBS and the suspensions were then centrifuged at 3000 xg for 5-15 min and the supernatants replaced with PBS (pH 7). Incubation was made for 24 hours. Three replicates of each individual isolates were used. Controls experiments lacking the mouthwash products were also included. The bacterial samples were then ready to be investigated by scanning electron microscopy.

Quantification of Cell Free Endotoxin in Supernatants of the Exposed Bacteria Using the LAL Assay: The End-point microplate method was used (Pyrochrome® Cape Cod Incorporated, USA) [16]. Pyrochrome® is packaged in lyophilised form in a 3.2 ml $vial^{-1}$ fill size. It contains an aqueous extract of amoebocytes of *Limulus polyphemus*, dextran (stabiliser), EDTA, $CaCl_2$, $MgCl_2$, buffer and chromogenic substrate (Boc-Leu-Gly-Arg-p-nitroanilide).

Control standard endotoxin (*E. coli* 0113:H10); 4, 2, 1 and 0.5 Endotoxin Unit per ml (EU ml^{-1}) were prepared and controls lacking the mouthwash were included. Samples and standards were brought to room temperature and mixed vigorously for one minute using a Vortex mixer. LAL was reconstituted with 3.2ml of reconstitution buffer, swirled gently and kept, to rehydrate, at room temperature for 3-5 minutes prior use (this solution is stable for 3 hours of reconstitution if stored cold (2-8° or on ice).

Timing of the test began immediately the LAL was added to the first microplate; standards and samples (50 µl) were also added in microplate wells. LAL solution (50 µl) was added and mixed with the standard and the samples. After incubation at 37° C for 45 minutes, 50 µl of 50% v/v HCl solution was added and mixed immediately. Absorbance was read in a microplate reader at 405 nm.

The intensity of the yellow colour formed was then measured at 405 nm using a microplate reader and the level of free endotoxin was determined by reference to a standard curve (4-0. 5 EU ml^{-1}), prepared from a standard solution of endotoxin.

RESULTS

Scanning Electron Microscope Images Showing the Effect of the Mouthwash Products: Both Gram Negative bacteria (*E. coli*) and Gram Positive bacteria (*S. epidermidis*) were examined using the scanning electron microscope. The scanning electron micrograph of the non treated *E. coli* cells showed straight rods occur singly Figure (1a). On the other hand *E. coli* cells after treatment with all mouthwash products exhibited some degree of damage in their cell wall and also a decrease in the number of cells (Figure -1b, 1c and 1d). The scanning electron micrograph of non treated *S. epidermidis* bacterial cell showed spherical cells in irregular clusters (Figure 2a). On the other hand *S. epidermidis* cells after treatment with all mouthwash products exhibited some damage to their cell wall and a decrease in cell numbers (Figure -2b, 2c and 2d).

Quantification of Bacterial Cell Free Endotoxin in Supernatants Exposed to Mouthwash (Using LAL Assay): Exposure of bacteria to the mouthwash at different concentrations, over a 24 hours incubation period, led to liberation of bacterial endotoxin released from the exposed bacteria (Fig. 3a-3b). In the case of the Orasept, large amounts of bacterial endotoxins was released in contrast, a small libration of endotoxin was observed in case of Peppermint; intermediate endotoxins values followed Aloe treatment (Fig. 3a-3b).

DISCUSSION

This study was primarily designed to compare the efficacy of three mouthwashes against endotoxin producing bacterial isolates. As was commented upon in the Introduction the main reason for conducting the work reported here was to determine if mouthwash products are likely to have a positive or a negative impact, either in the host or on the oral cavity and environment, notably in relation to the effectiveness of treatment. Since the results of *in vitro*, animal and clinical studies support

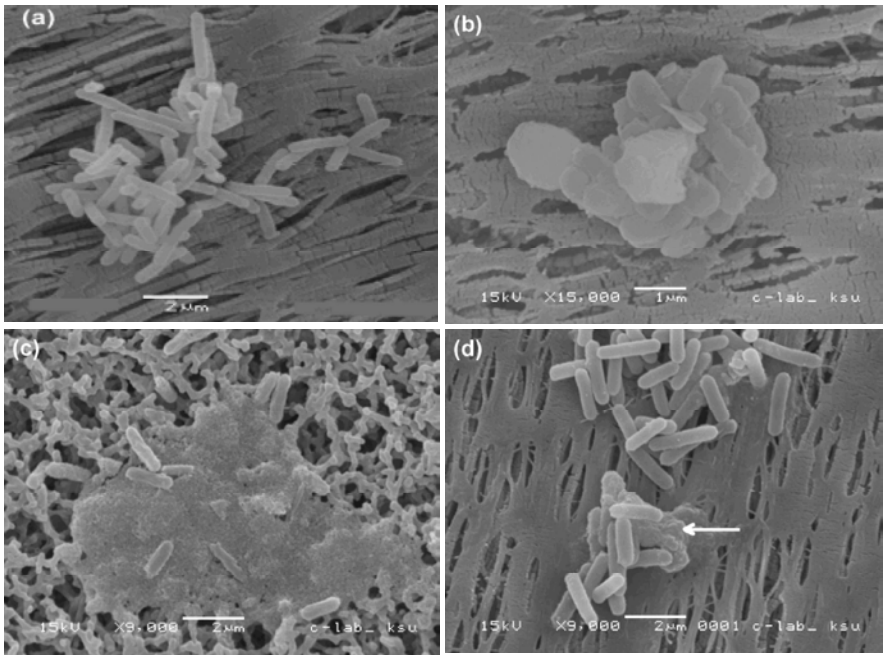

Fig. 1: Scanning Electron Micrograph of *E. coli* mounted on filter paper from oral cavity (SEM), (a) Non treated bacterial cell (NT) of *E. coli* showing straight rods occurring singly; (b) bacterial cell of *E. coli* treated with (Al), showing some damage to their cell wall and decreasing cell numbers. (c) Bacterial cell of *E. coli* treated with (Pe), showing some damage to their cell wall and a decrease in cell number. (d) Bacterial cell of *E. coli* treated with (Or), showing some damage to their cell wall and a decrease in the number of cells

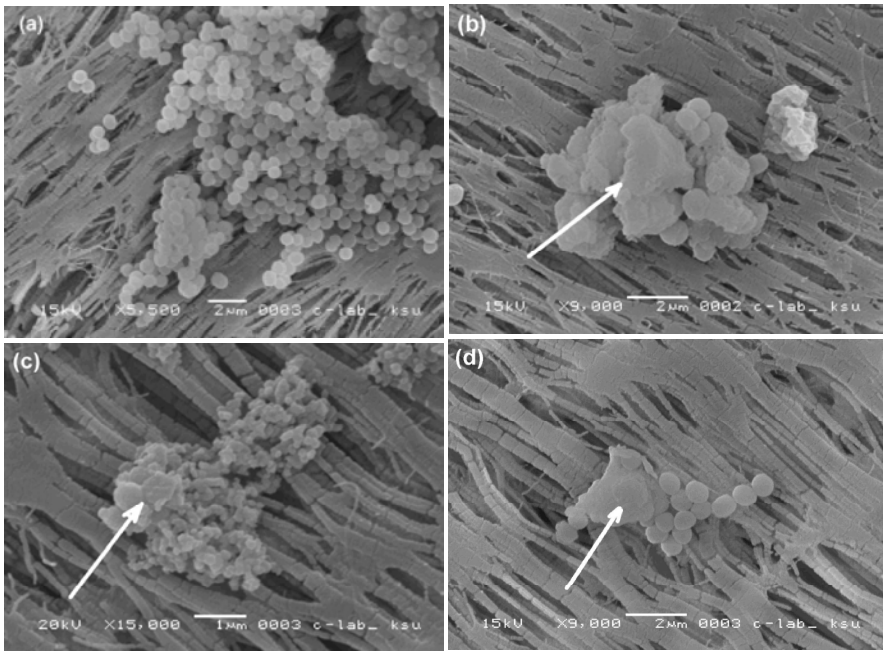

Fig. 2: Scanning Electron Micrograph of *S. epedermidis* mounted on filter paper from oral cavity (SEM), (a) Non treated bacterial cell (NT) of *S. epedermidis* before showed spherical cells in irregular cluters; (b) bacterial cell of *S. epedermidis* treated with (Al) which illustrated some damage in their cell wall and decreasing in number of cells. (c) Bacterial cell of *S. epedermidis* treated with (Pe), which illustrated some damage in their cell wall and decreasing in number of cells. (d) Bacterial cell of *S. epedermidis* treated with (Or), which illustrated some damage in their cell wall and decreasing in number of cells

Fig. 2: Scanning Electron Micrograph of *E. coli* mounted on filter paper from oral cavity (SEM), (a) Non treated bacterial cell (NT) of *S.epidermidis* showing straight rods occurring singly; (b) bacterial cell of *S.epidermidis* treated with (Al), showing some damage to their cell wall and decreasing cell numbers. (c) Bacterial cell of *S. epidermidis* treated with (Pe), showing some damage to their cell wall and a decrease in cell number. (d) Bacterial cell of *S.epidermidis* treated with (Or), showing some damage to their cell wall and a decrease in the number of cells

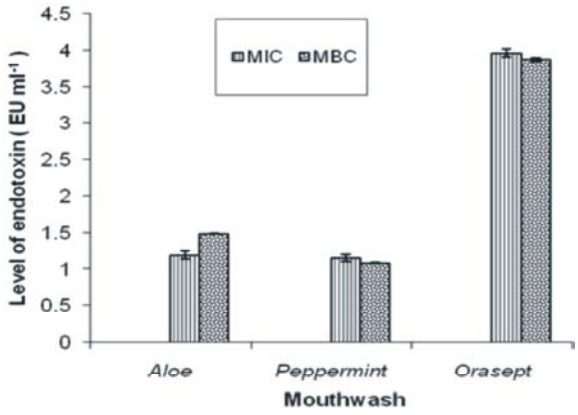

Fig. 3a: Quantification (using LAL assay) of the ability of mouthwash products to release bacterial endotoxin when *E.coli* was exposed for 24 hours to MIC and MBC. Means of triplicate, ± standard error

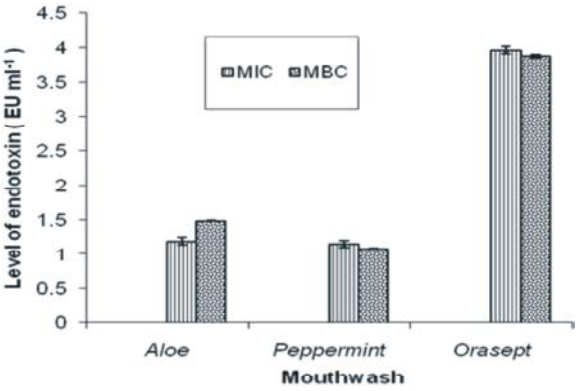

Fig. 3b: Quantification (using LAL assay) of the ability of mouthwash products to release bacterial endotoxin when *P. aeruginosa* was exposed for 24 hours to MIC and MBC. Means of triplicate, ± standard error

the hypothesis that endotoxin is liberated during antibiotic treatment of Gram-negative infections, it is important to consider the replacement of antibiotics with treatments that do not induce this effect.

Scanning electron microscopic studies using *E. coli,* as a typical Gram Negative bacterium and *S. epidermidis,* as a typical Gram Positive bacterium showed that non-treated *E. coli* cells occurred as single straight rods. Following treatment with all mouthwash products, damage was seen in all cells of this bacterium and numbers decreased. The scanning electron micrograph of non treated *S. epedermidis* bacterial cell showed spherical cells in irregular clusters (Figure 2a). While *S. epedermidis* cells after treatment with all mouthwash products exhibited some damage to their cell wall and decrease in cell number (Figure -2b, 2c and 2d). Fine *et al*., 2005 [17], using a simulated office visit model, showed that preprocedural use of an antimicrobial mouth rinse (Listerine) resulted in a 93.6 percent reduction in the number of viable bacteria in a dental aerosol produced by ultrasonic scaling.

In the current investigation, the *in vitro* libration of endotoxin has been induced with selected types of mouthwashes targeted toward, bacterial endotoxin. Mouthwashes having bacterial endotoxin as the main target vary in their ability to release endotoxin. Specific mouthwash have been classified as “weak” (peppermint), because a high concentrations is needed to kill or inhibit the growth of bacteria and conversely, “strong” (e.g. orasept) where only a low concentration is required; such a classification has been broadly confirmed by work reported here.

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