International Journal of Pharmaceutics, 65 (1990) 127–132
Elsevier

IJP 02207

The synergistic effect of direct and indirect ultrasonic energy and chlorhexidine gluconate on spores of *Bacillus subtilis*

S.P. Gorman, D.S. Jones * and A.M. Loftus

School of Pharmacy, Medical Biology Centre, The Queen's University of Belfast, Belfast BT9 7BL (U.K.)

(Received 5 May 1990)
(Accepted 6 June 1990)

Key words: Chlorhexidine; Ultrasonic energy; Bacterial spore; Sporicidal; *Bacillus subtilis*; Synergism

Summary

Aqueous chlorhexidine gluconate (5 and 10% w/v) in combination with direct ultrasonic energy from an ultrasonic disintegrator linked to a cooling system (20 °C) decreased the viability of *Bacillus subtilis* spore suspensions (1×10^6 cfu/ml) to levels of 47.7 and 4.8%, respectively, after 3 h contact. No viable spores were detected after 3 h with 10% chlorhexidine in 50% isopropyl alcohol. These solutions exhibited no sporicidal activity at 20 °C in the absence of ultrasonic energy. Spore viability was not greatly reduced (72% viable) when sonication was performed at 20 °C over 3 h. When temperature was allowed to rise naturally in an ultrasonic bath (65 °C attained at 3 h) no viable spores were detected following 3 h indirect sonication and spore contact with alcoholic 10% chlorhexidine in glass bottles. Marked improvements in sporicidal activity were observed with each formulation when spores were indirectly sonicated in a thin rubber membrane container. Aqueous and alcoholic 10% chlorhexidine achieved total spore kill at 3 h whilst a 99.97% reduction in viability was apparent by 2 h (43 °C) with the alcoholic solution.

Introduction

Chlorhexidine gluconate is an antimicrobial agent exhibiting excellent activity at low concentration against Gram-positive bacteria. Gram-negative bacteria are somewhat less sensitive whilst activity against bacterial spores is negligible at ambient temperature (Scott and Gorman, 1987). The compound is used extensively as a disinfectant, antiseptic and preservative in concentrations ranging from 0.001% to as high as 4.0% w/v. Although considered to possess no useful sporicidal activity, chlorhexidine gluconate (1.0%) is known to effect a reduction in spore viability of 99.999% in 6 min at a temperature of 98–100 °C (Anon., 1981). Shaker et al. (1986) showed chlorhexidine acetate (0.0025% w/v) to inhibit spore outgrowth but not germination. This concentration was not sporicidal at temperatures up to 37 °C though killing of *Bacillus subtilis* spores occurred at higher temperatures. Chlorhexidine gluconate (2% w/v) at room temperature required 6 days to exert a kill of 96% on spores of *B. subtilis* (Sykes, 1970). We have demonstrated (Gorman et al., 1987) much improved sporicidal activity in chlorhexidine gluconate solutions by addition of isopropyl alcohol. No viable spores of

Correspondence: S.P. Gorman, School of Pharmacy, Medical Biology Centre, The Queen's University of Belfast, Belfast BT9 7BL, U.K.
* *Present address*: Department of Pharmacy, University of Otago Medical School, Dunedin, New Zealand.

B. subtilis were recoverable after 3 h contact at 55 °C with the alcoholic chlorhexidine solution (10% w/v) and none after 4 h with the aqueous solution at this temperature.

We have previously shown that sporicidal activity of antimicrobial agents may be potentiated by application of ultrasound (Gorman et al., 1983a). This study examines the effect of ultrasound in the presence and absence of elevated temperatures on the sporicidal activity of chlorhexidine gluconate.

Materials and Methods

Preparation of spores

Spores of *B. subtilis* NCTC 10073 were produced as described by Gorman et al. (1987). Briefly, spores were grown on the medium of Beeby and Whitehouse (1965) by incubation at 30 °C for 5 days. Spores were harvested, washed four times with sterile distilled water and incubated in phosphate buffer (0.066 M, pH 7) containing 200 μg/ml lysozyme for 60 min at 37 °C. Following two further washes in sterile distilled water the spores were stored in dense suspensions at 4 °C.

Chemicals and materials

Chlorhexidine gluconate (20% w/v) solution was obtained from ICI Pharmaceuticals. Histidine, lecithin and polysorbate 80 were supplied by Sigma and asolectin by Fluorochem. Membrane filters (0.22 μm pore diameter, Millipore Ltd) were employed.

Germination medium

This was used as part of the chlorhexidine inactivation procedure to enhance revival of sublethally injured spores and is described by Gorman et al. (1987).

Determination of sporicidal activity

A 9.9 ml volume of chlorhexidine gluconate, equilibrated at the appropriate temperature, was added to 0.1 ml of standardised spore suspension (approx. 1×10^8 viable spores/ml). At timed intervals, 1 ml samples of the mixture were withdrawn and processed via the inactivation systems described by Gorman et al. (1987) prior to plating on Tryptone Soya agar (Oxoid). Agar plates were incubated at 32 °C for a minimum of 72 h and the number of cfu/ml determined. Inactivation of chlorhexidine required membrane filtration of the undiluted and serially diluted samples removed at the timed intervals. Membranes were washed several times with germination medium and transferred to agar containing asolectin, lecithin and histidine as chemical inactivators.

Ultrasonication

Two types of apparatus were used to provide ultrasonic energy. An MSE Soniprep 150 W ultrasonic disintegrator fitted with a titanium probe (tip diameter 9.5 mm, transformation ratio 55 : 1) operating at a nominal frequency of 23 kHz was used to impart direct ultrasonic energy to the bacterial spore suspensions. The probe was sterilised by dry heat before use. Spore suspensions, in chlorhexidine gluconate, were sonicated at an amplitude of 28 μm (36.81 W s min^{-1}). The spore-chlorhexidine mixture was contained in a sealed, jacketed cell connected to a Frigomix (Braun) containing propylene glycol coolant. Temperature was maintained at a constant 20 °C by a continuous flow through system.

A 150 W ultrasonic bath (Dawe Sonicleaner) provided ultrasonic energy indirectly to spore suspensions in either glass bottles or thin rubber membrane containers. This operated at a nominal frequency of 50 Hz and pulsed at twice the supply frequency. Glass, screw-cap, McCartney bottles having a wall thickness of 2 mm were used to contain the spore-chlorhexidine mixture for sonication. These were arranged on the floor of the ultrasonic bath to produce minimum disturbance of the ultrasonic waves. The volume of water in the bath was maintained at a set level above the fluid level in the McCartney bottles.

Additionally, spores in the presence or absence of chlorhexidine were contained in thin rubber finger stalls. A rubber bung with a central inlet/sample port was used to seal the thin rubber containers which were suspended in the water-filled ultrasonic bath, as described in Fig. 1. The

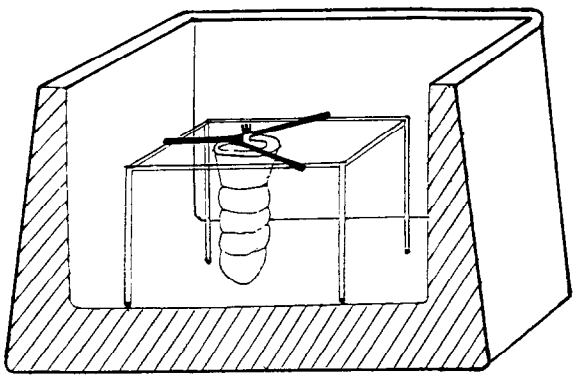

Fig. 1. Cross-section of the ultrasonic bath showing position of the thin rubber finger stall. A rubber bung with a central inlet/sample port is inserted into the finger stall. The water level in the bath is maintained above that of the spore-chlorhexidine mixture (10 ml) in the rubber finger stall.

integrity of the rubber was checked by investigation of leakage of included amaranth dye from the finger stall over a period of several hours ultrasonication. At appropriate time intervals samples of the spore-chlorhexidine mixture were removed via the sample port and inactivated, as described, prior to incubation and enumeration.

Results and Discussion

The majority of antibacterial agents lack significant sporicidal activity at ambient or intermediate range temperatures (37–70 °C). Chlorhexidine has previously been shown by us (Gorman et al., 1987) to exhibit significant sporicidal activity when employed at a concentration of 10% w/v in the presence of 50% isopropyl alcohol and at temperatures within this quoted range. Further advantage would be gained from such useful sporicidal activity being available at 20 °C. An ultrasonic disintegrator linked to a cooling system provided direct ultrasonic wave energy at this temperature by immersion of the ultrasonic probe in the suspensions under study. Concentrations of 5 and 10% w/v chlorhexidine in this system decreased the viability of spore suspensions, containing approx. 1×10^6 cfu/ml, to levels of 47.7 and 4.8%, respectively, after 3 h contact (Table 1). No decrease in spore viability was observed over 3 h at 20 °C with these chlorhexidine concentrations when ultrasonic energy was not applied. Table 1 also shows that addition of 50% isopropyl alcohol to chlorhexidine further increases sporicidal activity. No viable spores were detected after 3 h contact in this system with 10% chlorhexidine in alcoholic solution. This contrasts with the absence of sporicidal activity of the latter formulation in the absence of ultrasonic energy at 20 °C. In agreement with Gorman et al. (1983a), spore viability was not greatly reduced (72% viable) when sonication was performed at 20 °C over 3 h.

Sporicidal activity of chlorhexidine solutions was also enhanced by the indirect application of ultrasonic energy derived from an ultrasonic bath. Fig. 2a shows the decrease in viability of a spore suspension, in glass bottles, to levels of 6 and 0.06% over 3 and 5 h contact periods, respectively,

TABLE 1

The effect of direct ultrasonication at constant temperature (20 °C) on the activity of aqueous and alcoholic chlorhexidine gluconate against spores of Bacillus subtilis

Time of contact (h)	% Surviving spores [a] following direct ultrasonication [b] at 20 °C with			
	5% chlorhexidine	5% chlorhexidine in 50% isopropyl alcohol	10% chlorhexidine	10% chlorhexidine + 50% isopropyl alcohol
0	100	100	100	100
1	100	52.7	59.1	11.5
2	81.8	20.0	10.5	2.93
3	47.7	1.30	4.8	NDU [a]

[a] Initial viable count approx. 1×10^6/ml; NDU, no detectable units; inactivation carried out as described in Materials and Methods.
[b] Direct probe sonication of chlorhexidine-immersed spores at an amplitude of 26 μm in an ultrasonic disintegrator. Constant temperature maintained via link to cooling apparatus.

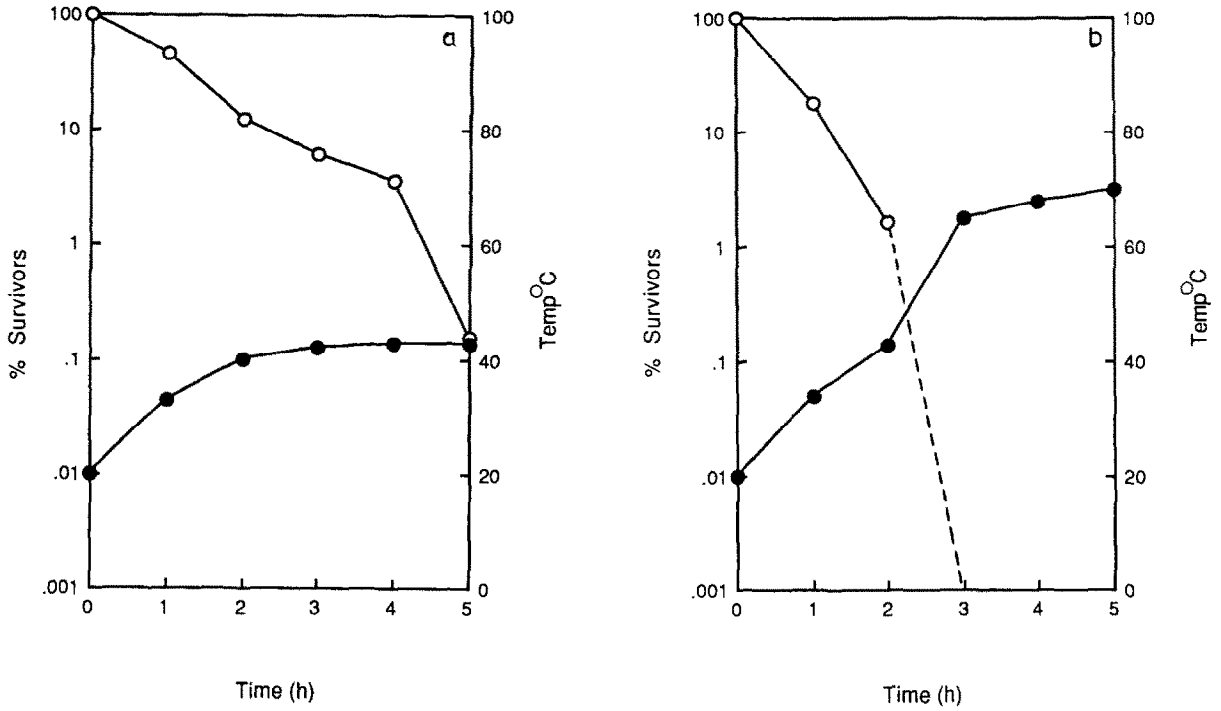

Fig. 2. The synergistic effect of ultrasonic energy derived from an ultrasonic bath and 10% chlorhexidine gluconate in 50% isopropyl alcohol on spores of *Bacillus subtilis* (initial viable spore count approx. 1×10^6/ml). (a) Ultrasonic bath – lid; (b) ultrasonic bath + lid. (●) Temperature (in °C); (○) % survivors; (------) no detectable units.

with 10% chlorhexidine in alcohol. The temperature of the system, including the spore-chlorhexidine mixture, is observed to rise from 20 to 43°C over this period. When a lid is placed on the ultrasonic bath to provide a closed system the temperature rises, due to ultrasonication, from 20

TABLE 2

Percentage surviving Bacillus subtilis spores in glass bottles or rubber membrane containers following contact with aqueous or alcoholic chlorhexidine gluconate and indirect sonication in an ultrasonic bath

Time of contact (h)	Temperature (°C)	% Surviving spores [a] following ultrasonication (in bath) [b]			
		10% chlorhexidine gluconate		10% chlorhexidine in 50% isopropyl alcohol	
		Glass bottle	Rubber membrane	Glass bottle	Rubber membrane
0	20	100	100	100	100
1	34	64.6	31.3	17.8	2.61
2	43	4.42	1.03	1.64	0.03
3	65	0.99	NDU [a]	NDU	NDU
4	68	NDU	NDU	NDU	NDU

[a] Initial viable spore count approx. 1×10^6 ml. NDU, no detectable units; inactivation carried out as described in Materials and Methods.

[b] Indirect sonication of chlorhexidine-immersed spores in an ultrasonic bath + lid. Temperature allowed to rise naturally over 4 h period of sonication.

to 70 °C over a 5 h period (Fig. 2b). Spore viability in such a system, in contrast with the temperature-controlled ultrasonic probe system, was reduced to a level of 80.7%. When spores were suspended in 50% alcohol and sonicated for 5 h in the closed bath, viability was reduced to 77.8%. No viable spores were detected following 3 h spore contact with 10% chlorhexidine in alcoholic solution when sonicated in this closed system. The temperature after this time was 65 °C.

Boucher (1979) found that the thickness of glass test tubes used to contain liquids in an ultrasonic bath contributed to a drop in the transmitted acoustic energy. Glass bottles were, therefore, substituted with impermeable thin rubber membranes to contain the spore-chlorhexidine mixtures to be sonicated in the closed bath system (Fig. 1). Table 2 shows the comparative effectiveness of aqueous and alcoholic chlorhexidine solutions in each of the containers. Marked improvements in sporicidal activity are observed with each formulation when examined in the rubber membrane container. Maximum sporicidal activity is displayed by the alcoholic solution with no survivors detected at 3 h. The internal surface of the rubber container was examined to ensure that no spores were adhered therefore depleting the viable spore count in suspension.

The mechanism underlying the synergism between chlorhexidine and ultrasonic energy evident in this study is of interest. We have previously found the protein-rich, multilayered spore coats of *B. subtilis* to provide a protective barrier against aqueous and alcoholic sodium hypochlorite combinations (Gorman et al., 1983b, 1984a), glutaraldehyde (Gorman et al., 1984b) and aqueous and alcoholic iodophor (Gorman et al., 1985). Recently, Shaker et al. (1988a,b) considered chlorhexidine acetate resistance of spores of *B. cereus* as possibly being due to the spore coat(s). Boucher (1979), in describing the effect of ultrasonication on the bacterial spore, suggested that cavitation shock waves produced would alter or partially destroy the layered outer coats and this provide access for the antimicrobial agent to the spore cortex and core. As the preferential site of chlorhexidine action in the bacterial cell is at the cytoplasmic membrane (Chawner and Gilbert, 1989), it appears likely that chlorhexidine must penetrate the bacterial spore to the level of the core to achieve kill. Additionally, the apparent 'softening' of the spore coat by contact with alcohol (Coates and Death, 1978; Gorman et al., 1987) would be expected to allow penetration of chlorhexidine in the presence of ultrasonic energy.

There have been several reports of the potentiation of antimicrobial activity by ultrasonic energy. Ahmed and Russell (1975) stated that ultrasonic waves alone are not sporicidal though these workers found synergism between ultrasonic energy and hydrogen peroxide in the killing of *B. cereus* spores, among other microorganisms. Earlier, Shaner (1964) reported beneficial outcome from the application of ultrasound and benzalkonium chloride or iodophor to the disinfection of surgical instruments. Further, Sierra and Boucher (1971) showed that suspensions of *B. subtilis* spores were non-viable after sonication in 1% w/v glutaraldehyde solution for 5 min whereas sterilisation with the agent alone required 60 min treatment.

The present study shows that chlorhexidine gluconate, particularly in isopropyl alcohol solution, exhibits considerable sporicidal activity when used in conjunction with ultrasonic energy. This activity could find use in the increasing number of situations where 'cold' liquid chemosterilisation of delicate or thermolabile materials and instruments is required.

References

Ahmed, F.I.C. and Russell, C., Synergism between ultrasonic waves and hydrogen peroxide in the killing of microorganisms. *J. Appl. Bacteriol.*, 39 (1975) 31–40.

Anon., Factors affecting Hibitane. In *ICI Antiseptics in General Practice*, ICI Pharmaceuticals Division, 1981, pp. 51–52.

Beeby, M.M. and Whitehouse, C.E., A bacterial spore test piece for the control of ethylene oxide sterilisation. *J. Appl. Bacteriol.*, 28 (1965) 349–360.

Boucher, R.M.G., Ultrasonics: a tool to improve biocidal efficacy of sterilants or disinfectants in hospital and dental practice. *Can. J. Pharm. Sci.*, 14 (1979) 1–12.

Chawner, J.I. and Gilbert, P., A comparative study of the bactericidal and growth inhibitory activities of the bis-

biguanides alexidine and chlorhexidine. *J. Appl. Bacteriol.*, 66 (1989) 243–252.

Coates, D. and Death, J.E., Sporicidal activity of mixtures of alcohol and hypochlorite. *J. Clin. Pathol.*, 31 (1978) 148–152.

Gorman, S.P., Scott, E.M., Hutchinson, E.P. and McDermott, L.M., Death, injury and revival of chemically-treated *Bacillus subtilis* spores. *J. Appl. Bacteriol.*, 54 (1983a) 91–99.

Gorman, S.P., Scott, E.M. and Hutchinson, E.P., The effect of sodium hypochlorite-methanol combinations on spores and spore forms of *Bacillus subtilis*. *Int. J. Pharm.*, 17 (1983b) 291–298.

Gorman, S.P., Scott, E.M. and Hutchinson, E.P., Hypochlorite effects on spores and spore forms of *Bacillus subtilis* and a spore lytic enzyme. *J. Appl. Bacteriol.*, 56 (1984a) 295–303.

Gorman, S.P., Scott, E.M. and Hutchinson, E.P., Interaction of the *Bacillus subtilis* spore protoplast, cortex, ion exchange and coatless forms with glutaraldehyde. *J. Appl. Bacteriol.*, 56 (1984b) 95–102.

Gorman, S.P., Scott, E.M. and Hutchinson, E.P., Effects of aqueous and alcoholic povidone-iodine on spores of *Bacillus subtilis*. *J. Appl. Bacteriol.*, 59 (1985) 99–105.

Gorman, S.P., Jones, D.S. and Loftus, A.M., The sporicidal activity and inactivation of chlorhexidine gluconate in aqueous and alcoholic solution. *J. Appl. Bacteriol.*, 63 (1987) 183–188.

Scott, E.M. and Gorman, S.P., Chemical disinfectants, antiseptics and preservatives. In Hugo, W.B. and Russell (Eds), *Pharmaceutical Microbiology*, 4th Edn, Blackwell, Oxford, 1987, pp. 226–253.

Shaker, L.A., Russell, A.D. and Furr, J.R., Aspects of the action of chlorhexidine on bacterial spores. *Int. J. Pharm.*, 34 (1986) 51–56.

Shaker, L.A., Furr, J.R. and Russell, A.D., Mechanism of resistance of *Bacillus subtilis* spores to chlorhexidine. *J. Appl. Bacteriol.*, 64 (1988a) 531–539.

Shaker, L.A., Dancer, B.N., Russell, A.D. and Furr, J.R., Emergence and development of chlorhexidine resistance during sporulation of *Bacillus subtilis* 168. *FEMS Microbiol. Lett.*, 51 (1988b) 73–76.

Shaner, E.O., Augmentation of bacterial action of germicides with ultrasound. *J. Acoust. Soc. Am.*, 36 (1964) 2238–2239.

Sierra, G. and Boucher, R.M.G., Ultrasonic synergistic effects in liquid phase chemical sterilisation. *Appl. Microbiol.*, 22 (1971) 160–164.

Sykes, G., The sporicidal properties of chemical disinfectants. *J. Appl. Bacteriol.*, 33 (1970) 147–156.