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Abstract

OBJECTIVES:

To evaluate cytotoxicity and bactericidal effects of chloramine-T.

METHODS:

In vitro study of various concentrations and exposure times to preparations containing human fibroblasts or 1.5 x 10^8 colony forming units per milliliter (CFU/mL) of 3 gram-positive bacteria-*Staphylococcus aureus*, methicillin-resistant *S aureus*, and vancomycin-resistant *Enterococcus faecalis*-and 2 gram-negative bacteria-*Escherichia coli* and *Pseudomonas aeruginosa*-with and without fetal bovine serum present.

MAIN OUTCOME MEASURES:

Percentage reduction of bacterial growth and percentage of viable fibroblasts 48 hours after exposure.

RESULTS:

All gram-positive growth was reduced by 95% to 100%, regardless of dose, with or without serum. *E coli* (gram-negative; with/without serum) was reduced 94% to 100% at antiseptic concentrations of 300 and 400 ppm. At 200 ppm, *E coli* growth was fully inhibited without serum present and by 50% with serum. *P aeruginosa* (gram-negative) was not significantly affected under any conditions. At 100 and 200 ppm, cell viability remained greater than 90% under all experimental conditions. A 300-ppm, 3-minute exposure to chloramine-T resulted in cell viability of up to 70%, with longer exposures producing lower viabilities. Serum did not affect cell viability in any condition.

CONCLUSIONS:

In vitro, chloramine-T at 200 ppm for 5 to 20 minutes was effective against 3 virulent gram-positive bacteria without fibroblast damage. At 300 ppm and 3 and 5 minutes, 30% of fibroblasts were damaged and 95% to 100 % of *E coli* were inhibited, respectively.

Management of bacterial burden in wounds to avert infection that delays healing or causes wound deterioration may be addressed clinically by several interventions, including debridement, pulsed lavage with suction, negative pressure wound therapy, systemic antibiotics, or application of topical antibiotics or antiseptic agents. Hydrotherapy (whirlpool) treatments have traditionally been used to loosen and remove necrotic tissue or other nonviable debris. Despite a lack of strong evidence, it is believed by some that hydrotherapy treatments, even with antiseptic additives, do not decrease bacterial burden, but could cause cross contamination and/or injury to regenerating cells in open wounds.

For the chemical management of local wound infections, topical antibiotics are preferred over systemic antibiotics because it has been found that systemic antibiotics rarely reach adequate levels in the granulation tissue of chronic wounds to successfully control propagating superficial bacteria.[1] An advantage of topical antibiotics is that by selectively binding to chemical targets found in bacterial cell membranes, but not in human cells, antibiotics are cytotoxic to bacteria in the wound but do not harm host cells.[2] A disadvantage of many topical antibiotics is that they have a narrow spectrum of bacterial

species against which they are effective. In a chronic wound environment where polymicrobial colonization occurs, 2 or more topical antibiotics may be necessary to cover the spectral range of bacterial species present.[3] In addition, because repeated use of antibiotics in treating wound infections may lead to bacterial resistance, it has become necessary to restrict their utilization to conditions where they are absolutely needed and to avoid their use as a first-line intervention.[4]

Topical antiseptic agents can also be used in wounds to manage bacterial burden. Antiseptics are chemicals that destroy or inhibit the growth and development of microorganisms in or on living tissue. Unlike antibiotics that act selectively on one target in the bacterial cell, antiseptics can act on several targets at the same time, including the cell membrane, cytoplasmic organelles, and nucleic acids.[5] Because antiseptic agents exert their antimicrobial effects on different cell targets, development of bacterial resistance is improbable despite prevalent clinical use.[2,5] Some investigators have concluded that the reasonable use of antiseptics could help reduce the usage of antibiotics, preserving their benefits for clinically critical situations.[6] Although resistance toward antiseptics has been reported, it is to a significantly lesser extent than reported with antibiotic usage.[7] The cell membrane targets disabled by antiseptic agents have a broad spectrum of activity that includes bacteria, viruses, fungi, and protozoa.[8] Some broad-spectrum antiseptics can impact all types of bacteria found in wounds, including gram-positive, gram-negative, and even some antibiotic-resistant strains.[2] Several antiseptic types exist, including alcohols (ethanol), anilides (triclocarban), biguanides (chlorhexidine), chlorine, iodine and silver compounds, peroxygens, and quaternary ammonium compounds.[5] The most commonly used antiseptics in clinical practice today include acetate, alcohol, boric acid, chlorhexidine, chloramine-T, povidone-iodine, silver nitrate, silver sulfadiazine, and sodium hypochlorite.

The use of antiseptics in wounds causes some concern and is controversial. One concern is the reported cytotoxic effect on cells involved in wound healing (leukocytes, keratinocytes, and fibroblasts) in vitro.[9,10] Another is that antiseptic agents such as sodium hypochlorite are rapidly inactivated in the presence of human wound exudate, blood sera, or extracellular matrices.

The cytotoxic effect appears to be dose dependent. It has been demonstrated in vitro that several antiseptics in low concentrations are not cytotoxic while maintaining their antimicrobial effect.[11] In a comprehensive review, a group of recognized wound care practitioners concluded that some antiseptics display cytotoxic properties, but if used correctly, may be effective antimicrobial agents.[12] Although many antiseptic agents have shown toxicity to human fibroblasts in vitro, the authors' review found that most in vivo studies have not displayed significant delays in wound healing.[13-21] Bennett et al[22] demonstrated in vivo that several common antiseptics did not inherently impede wound healing and that some actually improved angiogenesis and fibroblast proliferation. Work by Lindfors[23] suggested that an antiseptic solution was more effective than saline solution alone when used as a wound cleanser to promote wound healing. In addition, Neider[24] concluded that when their rates of causing contact sensitization are compared, antiseptics are superior to topical antibiotics. The weight of this evidence suggests a more careful consideration of these long-held concepts. Some beliefs date to 1919 when Fleming[25] concluded that antiseptics are not as effective against bacteria that reside in wounds as they are against bacteria in vitro.

Chloramine-T (sodium *n*-chloro-*para*-toluene sulfonamide), first introduced by H. D. Dakin in 1916,[26] is an antiseptic that consists of chlorine attached to nitrogen in primary and secondary amine groups. When the antiseptic white powder is placed in water, the chlorine-nitrogen bonds are gradually cleaved, allowing chlorine to be slowly dissolved into the water.[26] Compared with an equimolecular solution of neutral sodium hypochlorite, the rate of fall in available chlorine from chloramine-T is slower and varies directly with serum concentration.[26] The slow release of chlorine allows chloramine-T more prolonged antiseptic activity. In addition, its antiseptic value is about 4 times greater than hypochlorites; it is more efficient in the presence of blood and is less irritating than sodium hypochlorite.[27] Chloramine-T (Chlorazene; Wisconsin Pharmacal Company, LLC, Jackson, WI) is a commercially available antiseptic agent that is frequently used as an additive to therapeutic whirlpool water to control microorganisms and reduce the risk of wound infection by cross contamination.[20]

The purpose of the present in vitro study was to evaluate chloramine-T with respect to 3 commonly held assumptions about antiseptic solutions. These assumptions are that this antiseptic solution: (1) is not powerful enough to kill bacteria, especially more virulent strains; (2) is cytotoxic at any concentration; and (3) loses its bactericidal effects or any other desirable properties in the presence of mammalian proteins. Specifically, the first objective was to determine the antibacterial effects of chloramine-T at different dilutions, temperatures, and exposure times on common and/or virulent wound pathogens, including *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *S aureus* (MRSA), *Pseudomonas aeruginosa*, and vancomycin-resistant *Enterococcus* (VRE) *faecalis* in vitro. The second objective was, under similar conditions, to evaluate the effects of chloramine-T on cultured human fibroblasts in vitro. Finally, the third objective was to assess whether any bactericidal or cytotoxic properties were affected by the presence or absence of mammalian proteins.

Materials and Methods

Exposure of bacterial pathogens to chloramine-T

Commercially available type strains of 3 gram-positive bacteria commonly isolated from infected wounds were selected for testing and obtained in lyophilized form from the American Type Culture Collection (ATCC; Manassas, VA): *S aureus* (ATCC 6538), MRSA (ATCC 33591), and VRE *faecalis* (ATCC 51299). Also chosen because of their potential as wound pathogens were 2 gram-negative bacteria: *E coli* (ATCC 8739) and *P aeruginosa* (ATCC 9027). Just prior to experimentation, the lyophilized pellets were reconstituted in trypticase soy broth (Remel, Lenexa, KS) and streaked for growth on trypticase soy agar (Remel). For each bacterium tested, 3 to 5 colonies of an 18- to 24-hour culture were inoculated into trypticase soy broth and incubated at 37°C until turbid. Using a0.5 McFarland standard, the suspension was adjusted to a concentration of approximately 1.5×10^8 colony forming units per milliliter (CFU/mL), the standard concentration used when testing bacteria for susceptibility to antimicrobial agents.[28]

Chloramine-T was diluted to concentrations of 200, 300, or 400 ppm with sterile tap water. Mixtures of 5 mL of the appropriate chloramine-T solution and 0.5 mL of standardized bacterial suspension were prepared in sterile test tubes. Each of these mixtures was incubated for 5, 10, 15, and 20 minutes at 3 water bath temperatures: 36°C, 38°C, or 40°C. The incubation times and water temperatures were selected based on clinical hydrotherapy treatment durations and water temperatures such as those

used during wound irrigation/cleansing with whirlpool and pulsed lavage with vacuum. At the end of the exposure period, the mixture was removed from the water bath and 5 mL of sterile 0.01% sodium thiosulfate were added to neutralize the antimicrobial effects of chloramine-T. Following neutralization, a sterile 1:100 calibrated inoculating loop was used to streak the mixture to trypticase soy agar. Plates were incubated at 35°C to 37.5°C for 18 to 24 hours and then observed by 2 different researchers for the presence of growth. The number of colonies was multiplied by the dilution factor of 100 to determine colony counts and, subsequently, percentage reduction in growth due to the action of chloramine-T was calculated. If the number of colonies was too large to count accurately, the experiment was repeated using a 1:1000 calibrated loop. If colonies were still too numerous to count, a visual comparison was made between the test plate and a growth control plate to estimate the percentage reduction in growth. Each experiment was performed in duplicate, with triplicate testing performed when disparate results were seen. The reported percentage reduction is an average of duplicate testing. Appropriate positive and negative controls were performed for each time, temperature, and chloramine-T concentration variance.

Because protein at a wound site can interfere with the antimicrobial effects of chlorine-based antiseptics, an in vitro determination of its effects on chloramine-T was included in this study. The test protocol previously described was repeated with the addition of 5% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Inc, Lawrenceville, GA). The serum was transferred with a 250-microliter sterile pipette to 5 mL chloramine-T, and the tube was mixed for 1 minute prior to adding 0.5 mL of the standardized bacterial suspension. The remainder of the experiment was performed as described above.

Exposure of fibroblasts to chloramine-T

Human foreskin adherent fibroblasts (CRL-2522; ATCC, Manassas, VA) were selected based on the absence of any known cellular defects and were obtained as frozen stock. The cells were maintained according to ATCC product guidelines. The cells were thawed and diluted in 10% minimal essential media (MEM) (Atlanta Biologicals, Inc, Lawrenceville, GA) with 10% heat-inactivated FBS and 0.25 mg/mL gentamicin (Cambrex, Inc, Walkersville, MD) to remove dimethyl sulfoxide. Cells were washed and diluted to a concentration of approximately 1 x 10^6/T-25 tissue culture flask, (Corning, Inc, Corning, NY) or 1.5 x 10^5/well of 6 well plates (Costar, Corning, Inc, NY) with 10% MEM. Flasks and plates were incubated in 5% carbon dioxide at 37°C to produce a confluent monolayer of cells. Cells were fed every 2 to 3 days with 10% MEM. When the surfaces of the flasks/wells were covered with viable fibroblasts, the cells were treated with 0.05% trypsin plus 0.53 mM ethylene diamine triacetic acid (EDTA; Mediatech, Inc, Herndon, VA) to detach from the plastic surface. Then they were split at a ratio of 1:2 with 10% MEM and reincubated. Splits of cells were continued until sufficient flasks/wells were available for testing.

Chloramine-T was dissolved to a concentration of 1000 ppm in sterile tap water (50 mg into 50 mL). The 1000-ppm chloramine-T solution was then diluted to concentrations of 100, 200, and 300 ppm with 10% MEM (MEM with 10% FBS) or MEM alone. Culture media was removed and then replaced with one of the test media with varying dilutions of either chloramine-T, chloramine-T with serum, or bleach, as well as appropriate controls. The negative control (no cell death) was 10% MEM or MEM alone, and the positive control (>70% cell death) was 20% ethanol in MEM or 10% MEM. Exposure

times to the test media were 20, 10, or 3 minutes, with the cultures incubated at 37°C. Selection of the exposure times was based on clinical hydrotherapy treatment durations such as those used during wound irrigation/cleansing with whirlpool and pulsed lavage with vacuum. After the exposure, the cells were rinsed 2 times with MEM, then 10% MEM was added to the flasks/wells and the cells were placed back into the incubator. Using an inverted microscope, morphology of the cells was assessed immediately and at 6, 24, and 48 hours after exposure to the test medium. Alterations in morphology were graded based on changes noted (detachment, stunting, or retraction of cytoplasm). Cytotoxicity was evaluated using 0.4% trypan blue stain[29] (Sigma-Aldrich, Inc, St. Louis, MO) at 48 hours after exposure.

When grown under the recommended conditions, the fibroblasts were elongated (approximately 300-500 microns in length) with clear cytoplasm and nuclei and nucleoli barely discernable with the inverted phase microscope (Figure 1A). Morphologic alterations noted during this study were that the cells would (1) slightly shorten the cytoplasmic extensions to 200-300 microns in length with some gaps in monolayer (Figure 1B), (2) have moderately shortened cytoplasm measuring between 100 and 200 microns in length (Figure 1C),(3) retraction of cytoplasm and "rounding" of cell shape to approximately 50-150 microns in size (Figure 1D), (4) rounded to a size of 40-60 microns in diameter with the cytoplasm barely visible (Figure 1E), and (5) detached and rounded up in clusters (Figure 1F). These morphologies were then rated as follows:

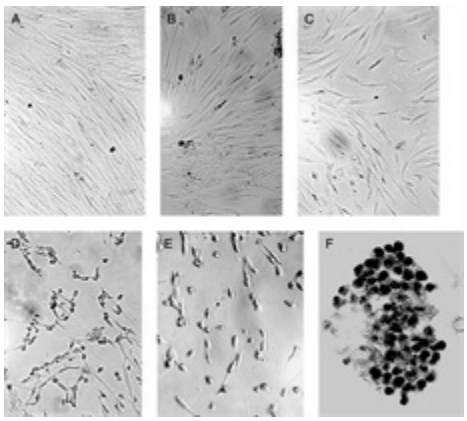

Figure 1: MORPHOLOGIC FEATURES OF HUMAN FIBROBLASTS AFTER EXPOSURE TO CHLORAMINE-T. DIGITAL PHOTOGRAPHS TAKEN FROM INVERTED MICROSCOPE, 100X MAGNIFICATION.

- no changes noted = 0
- 0-24% altered morphology = 1+
- 25%-49% altered morphology = 2+
- 50%-74% altered morphology = 3+
- ≥75% altered morphology = 4+.

Digital photographs taken from inverted microscope, 100× magnification. A. Normal morphology, human foreskin fibroblasts. B. Cells after exposure to chloramine-T: cells slightly shorter with some gaps in monolayer. C. Cells after exposure to chloramine-T: cells have shortened cytoplasmic extension (moderate). D. Cells after exposure to chloramine-T: note retraction of cytoplasm and "rounding" of

cell shape. E. Cells after exposure to chloramine-T: almost complete retraction of cytoplasm and "rounding" of cell shape. F. Nonviable cells: rounded, clustered and detached (trypan blue staining).

Viability was determined by trypan blue exclusion stain, where viable cells would not take up the stain but dead or damaged cells would. After the first observations were made, additional exposure times of 3 and 10 minutes were evaluated to assess the effects of chloramine-T on fibroblasts with and without serum.

Results

Bacterial pathogen response

Results of experiments on the 5 bacterial species are shown in Tables 1 through 3. The reported percentage reduction in growth is the average of results obtained from duplicate testing. Because chloramine-T was almost completely effective against all 3 of the gram-positive bacteria-*S aureus*, MRSA, and VRE *faecalis*- regardless of antiseptic concentration, water bath temperature, or time of exposure, the ranges of their results are combined in Table 1. The smallest percentage reduction in growth was 95%. Nearly all other results showed greater than 99.9% to 100% reduction in growth. The addition of 5% FBS to chloramine-T did not significantly alter the effectiveness of this antiseptic against gram-positive bacteria.

Table 1: EFFECTS OF 3 CHLORAMINE-T CONCENTRATIONS WITH AND WITHOUT EXPOSURE TO SERUM ON THE GROWTH OF *S AUREUS,* MRSA, AND VRE *FAECALIS* AT 3 TEMPERATURES AND 4 EXPOSURE TIMES

% Reduction of Growth with and Without 5% Serum			
	36°C	38°C	40°C
200 ppm Chloramine-T Exposure Times			
5 minutes	95 – >99.99	95 – >99.99	>99.9 – 100
10 minutes	99.9 – 100	98 – 100	>99.9 – 100
15 minutes	98 – 100	95 – 100	99.9 – 100
20 minutes	95 – >99.9	>99.9 – 100	>99.9 – 100
300 ppm Chloramine-T Exposure Times			
5 minutes	>99.9 – 100	>99.9 – 100	>99.9 – 100
10 minutes	>99.99 – 100	>99.9 – >99.99	>99.9 – 100
15 minutes	>99.9 – >99.99	>99.9 – 100	>99.9 – 100
20 minutes	>99.9 – 100	>99.9 – 100	>99.9 – 100
400 ppm Chloramine-T Exposure Times			
5 minutes	>99.9 – 100	>99.9 – 100	>99.9 – 100
10 minutes	>99.99 – 100	>99.9 – 100	>99.9 – 100
15 minutes	>99.9 – 100	99.9 – 100	>99.99 – 100
20 minutes	>99.9 – 100	>99.9 – 100	>99.9 – 100

Table 2: EFFECTS OF 3 CHLORAMINE-T CONCENTRATIONS WITH AND WITHOUT EXPOSURE TO SERUM ON THE GROWTH OF *E COLI* AT 3 TEMPERATURES AND 4 EXPOSURE TIMES

% Reduction of Growth				**% Reduction of Growth With 5% Serum Added**			
	36°C	38°C	40°C		36°C	38°C	40°C
200 ppm Chloramine-T Exposure Times				**200 ppm Chloramine-T Exposure Times**			
5 minutes	95	100	>99.9	5 minutes	50	50	30
10 minutes	99.3	>99.99	>99.9	10 minutes	50	50	30
15 minutes	99.6	>99.99	>99.9	15 minutes	50	50	50
20 minutes	99.6	100	>99.9	20 minutes	50	50	50
300 ppm Chloramine-T Exposure Times				**300 ppm Chloramine-T Exposure Times**			
5 minutes	>99.9	99.9	>99.99	5 minutes	>99.9	94	>99.9
10 minutes	99.9	>99.9	>99.99	10 minutes	100	95	>99.9
15 minutes	>99.9	99.9	>99.99	15 minutes	100	98	>99.9
20 minutes	99.9	99.9	>99.9	20 minutes	>99.99	96	>99.9
400 ppm Chloramine-T Exposure Times				**400 ppm Chloramine-T Exposure Times**			
5 minutes	100	100	100	5 minutes	>99.9	>99.9	100
10 minutes	100	100	100	10 minutes	>99.9	100	100
15 minutes	>99.99	100	100	15 minutes	>99.99	>99.9	>99.9
20 minutes	100	>99.99	100	20 minutes	>99.9	100	100

Table 3: EFFECTS OF 3 CHLORAMINE-T CONCENTRATIONS WITH AND WITHOUT EXPOSURE TO SERUM ON THE GROWTH OF *P AERUGINOSA* AT 3 TEMPERATURES AND 4 EXPOSURE TIMES

% Reduction of Growth				**% Reduction of Growth With 5% Serum Added**			
	36°C	38°C	40°C		36°C	38°C	40°C
200 ppm Chloramine-T Exposure Times				**200 ppm Chloramine-T Exposure Times**			
5 minutes	5	5	30	5 minutes	10	5	10
10 minutes	5	10	30	10 minutes	25	0	10
15 minutes	5	20	30	15 minutes	15	0	10
20 minutes	10	40	30	20 minutes	30	5	5
300 ppm Chloramine-T Exposure Times				**300 ppm Chloramine-T Exposure Times**			
5 minutes	0	25	0	5 minutes	0	0	0
10 minutes	5	15	5	10 minutes	0	0	0
15 minutes	5	15	20	15 minutes	0	0	0
20 minutes	5	20	25	20 minutes	0	0	0
400 ppm Chloramine-T Exposure Times				**400 ppm Chloramine-T Exposure Times**			
5 minutes	0	50	15	5 minutes	0	0	10
10 minutes	0	40	30	10 minutes	0	0	5
15 minutes	5	60	40	15 minutes	0	0	5
20 minutes	5	60	65	20 minutes	0	0	10

Chloramine-T was also highly effective against *E coli* (Table 2). At 200 ppm and 36°C, chloramine-T caused 95% to 99.6% reduction in *E coli* growth, the smallest reduction observed for this bacterium. At all other temperatures, exposure times, and concentrations, chloramine-T reduced *E coli* growth by 99.9% or more. The addition of 5% serum had little to no effect on chloramine-T at concentrations of 300 to 400 ppm; however, the serum significantly affected the bactericidal action of chloramine-T at 200 ppm, at all temperatures and exposure times. Without serum, chloramine-T caused a 95% to 100% reduction of *E coli* growth, but when serum was added to the antiseptic, *E coli* growth was reduced by only 30% to 50%.

As shown in Table 3, chloramine-T demonstrated little effectiveness in reducing the growth of *P aeruginosa*. Under most experimental conditions, chloramine-T caused a reduction of less than 40% for this bacterium. Only at a concentration of 400 ppm and temperatures of 38°C and 40°C did chloramine-T cause 50% or more reduction in growth for several of the exposure times. The addition of serum had profound influence on the effectiveness of chloramine-T on *P aeruginosa*. At most exposure times, temperatures, and concentrations, chloramine-T with serum caused no reduction in the growth of *P aeruginosa*.

Fibroblast response

The results of the experiments on the human fibroblasts used in this study are shown in Tables 4 through 7. The observed alterations in morphology results were obtained from at least triplicate testing. Results of experiments with and without the addition of 10% FBS to the chloramine-T were combined and averaged because there were no significant differences in the changes observed. The negative controls (MEM or 10% MEM) had no changes in their morphology during the various timings and had good viability (>90%) in each of the experiments. The positive controls (20% ethanol in MEM or 10% MEM) displayed significantly altered morphology under all conditions of the experiment, and all displayed less than 10% viability at the end of the testing.

Table 4: EFFECTS OF CHLORAMINE-T AT A CONCENTRATION OF 100 PPM WITH AND WITHOUT ADDITION OF SERUM ON THE MORPHOLOGY AND VIABILITY OF HUMAN FIBROBLASTS

	Immediate	**6 hours**	**24 hours**	**48 hours**	**Viability With Trypan Blue Staining After 48 Hours**
100 ppm Chloramine-T Exposure Times					
3 minutes	0	0	0	0	>90%
10 minutes	0-1+	0	0	0	>90%
20 minutes	1+	0	0	0	>90%
Negative control	0	0	0	0	>90%
Positive control	4+	4+	4+	4+	<10%

0 = no changes; 1+ = 0-24% altered; 4+ = >75% altered.

Table 5: EFFECTS OF CHLORAMINE-T AT A CONCENTRATION OF 200 PPM WITH AND WITHOUT ADDITION OF SERUM ON THE MORPHOLOGY AND VIABILITY OF HUMAN FIBROBLASTS

	Immediate	**6 hours**	**24 hours**	**48 hours**	**Viability With Trypan Blue Staining After 48 Hours**
200 ppm Chloramine-T Exposure Times					

3 minutes	2+	0-1+	0	0	>90%
10 minutes	4+	2+	1+	0	>90%
20 minutes	4+	4+	3+	0	>90%
Negative control	0	0	0	0	>90%
Positive control	4+	4+	4+	4+	<10%

0 = no changes; 1+ = 0-24% altered; 2+ = 25-49% altered; 3+ = 50-74% altered; 4+ = >75% altered.

Table 6: EFFECTS OF CHLORAMINE-T AT A CONCENTRATION OF 300 PPM WITH AND WITHOUT ADDITION OF SERUM ON THE MORPHOLOGY AND VIABILITY OF HUMAN FIBROBLASTS

	Immediate	**6 hours**	**24 hours**	**48 hours**	**Viability With Trypan Blue Staining After 48 Hours**
300 ppm Chloramine-T Exposure Times					
3 minutes	3+	2-3+	3+	2+	-70%
10 minutes	4+	3+	3+	2-3+	-40%
20 minutes	4+	4+	4+	4+	<10%
Negative control	0	0	0	0	>90%
Positive control	4+	4+	4+	4+	<10%

0 = no changes; 1+ = 0-24% altered; 2+ = 25-49% altered; 3+ = 50-74% altered; 4+ = z75% altered.

Table 7: EFFECTS OF BLEACH AT 4 CONCENTRATIONS WITH AND WITHOUT ADDITION OF SERUM ON THE MORPHOLOGY AND VIABILITY OF HUMAN FIBROBLASTS AT 20-MINUTE EXPOSURES

	6 hours	**24 hours**	**48 hours**	**Viability With Trypan Blue Staining After 48 Hours**
50 ppm bleach	3+	0	0	NT
100 ppm bleach	4+	0	0	NT
200 ppm bleach	4+	4+	3+	<50%
400 ppm bleach	4+	4+	4+	<10%
Negative control	0	0	0	>90%
Positive control	4+	4+	4+	<10%

0 = no changes; 1+ = 0-24% altered; 2+ = 25-49% altered; 3+ = 50-74% altered; 4+ = z 75% altered; NT = not tested.

No significant morphologic changes were observed in the cultured human fibroblasts at a concentration of 100 ppm of chloramine-T, regardless of the time of exposure (Table 4). Minimal morphologic alterations were seen immediately after the chloramine-T had been removed from the cells, but by the 6-hour observation time, the cells had returned to their normal morphology. This morphology continued throughout the remaining observations. At all 3 exposure times, cells demonstrated greater than 90% viability after chloramine-T exposure.

Cells exposed to 200 ppm chloramine-T were observed to have more significant morphologic alterations (Table 5). With a 20-minute exposure, the cells had considerable changes in shape and size immediately following contact with chloramine-T. The fibroblasts did not return to their normal morphology until 48 hours from the time of exposure (Figure 2). At the 10-minute exposure time, a substantial amount of morphologic alterations was seen immediately after exposure. The cells showed increased recovery within 24 hours. A 3-minute exposure to chloramine-T had fewer changes when compared with 10- and 20-minute exposures; the cells returned to their preexposure form within 24

hours. As with the 100-ppm concentration, the 3-, 10-, and 20-minute exposures demonstrated greater than 90% viability by trypan blue 48 hours after their exposure to the chloramine-T.

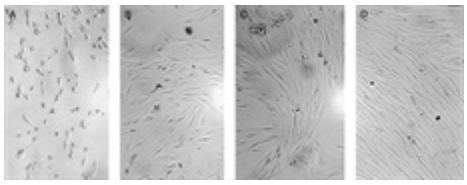

Figure 2: MORPHOLOGIC RECOVERY FOLLOWING 20-MINUTE EXPOSURE TO CHLORAMINE-T AT 200 PPMA. Human fibroblasts immediately after exposure to chloramine-T at 200 ppm for 20 minutes. B. Fibroblasts 6 hours after exposure: cells show slight elongation and widening of cytoplasm. C. 24 hours after exposure: cells show moderate elongation of cytoplasm. D. Cells return to original morphology 48 hours after exposure to chloramine-T.

Chloramine-T, at a concentration of 300 ppm, caused major morphologic alterations (Table 6). The 20-minute exposure time resulted in fibroblasts rounding up immediately after exposure; the cells were not able to return to their normal morphology by 48 hours. When tested with trypan blue, these cells demonstrated less than 10% viability. After a 10-minute exposure at this concentration, considerable changes to cell morphology were noted immediately. Limited recovery of the cells' morphology was observed at 24 and 48 hours, with the viability approximately 40%. The 3-minute exposure time, although causing some significant alterations in morphology in the immediate stage, allowed a significant number of cells to return to normal or near-normal morphology. At 48 hours, these cells were approximately 70% viable by trypan blue stain.

The fibroblasts were also tested against 4 different concentrations of bleach with a 20-minute exposure (Table 7). All 4 dilutions showed significant changes in morphology at 6 hours. The 50- and 100-ppm cultures returned to normal morphology after 24 hours. The 200- and 400-ppm cultures continued to have altered morphologies. The 200-ppm culture demonstrated less than 50% viability, and the 400-ppm culture had less than 10% viability.

Discussion

The results of this study show that chloramine-T, with or without serum, was nearly 100% bactericidal against the gram-positive bacteria tested, regardless of time of exposure, water bath temperature, or antiseptic concentration. These results are similar to reports by others who have found gram-positive bacteria intrinsically susceptible to many antibacterial agents due to cell walls that consist predominately of peptidoglycan and teichoic acids.[30] Enterococci are often less susceptible to biocides than are staphylococci[8]; however, the results of this study showed similar susceptibility to chloramine-T. It is interesting to note that antibiotic resistance associated with MRSA and VRE did not result in resistance to the effects of chloramine-T, although one study has suggested that VRE strains are just as susceptible to antiseptics as are antibiotic-susceptible strains.[31]

In general, gram-negative bacteria are intrinsically more resistant to biocides than are gram-positive bacteria; however, the gram-negative *E coli* is similar to the gram-positive *S aureus* in susceptibility to some antiseptics.[32] The results of this study also found that *E coli* and *S aureus* reacted similarly to

chloramine-T, except that *E coli* had increased resistance to 200 ppm chloramine-T when serum was added. The reason for this result is unknown.

Other gram-negative bacteria are highly resistant to biocides because their complex outer membrane acts as a barrier to antiseptics and disinfectants.[8] *P aeruginosa* is particularly resistant because the high magnesium concentration in its cell wall produces strong links between its lipopolysaccharides. Furthermore, its porin proteins are narrow and do not easily allow diffusion of molecules into the cell. The results of this study confirmed the biocide resistance of *P aeruginosa*. At all concentrations of chloramine-T, there was little to no reduction in growth of this bacterium, although the greatest antibacterial effect was seen with 400 ppm of chloramine-T. Once serum was added, the chloramine-T rarely had any effect on *P aeruginosa*, suggesting that chloramine-T may not be effective if this bacterium is colonizing a wound. Further testing is needed to study the effectiveness of chloramine-T against *P aeruginosa*, particularly at lower bacterial concentrations. The standard concentration used for testing the susceptibility of bacterial suspensions to antimicrobial agents, 1.5×10^8 CFU/mL, provides a conservative evaluation of susceptibility. It may not accurately consider the usual bacterial burden at an infected wound site, generally believed to be closer to 10^6 CFU/mL.[33] Subsequent testing against gram-negative organisms at a lower bacterial concentration may be warranted.

The present study also shows that the negative effect chloramine-T has on human fibroblasts in vitro is dependent on the concentration used and the time of the exposure. A concentration of 300 ppm was detrimental to the cells that were used under the experimental conditions described. After the cells were exposed to chloramine-T for 20 minutes, they were no longer viable after 48 hours. With a 10-minute exposure at that concentration, only 40% of the cells were viable. A 3-minute exposure had a lesser detrimental affect, leaving 70% of the cells still viable. At concentrations of 100 and 200 ppm, after undergoing some morphologic alterations, the cultured fibroblasts returned to their preexposure morphology and demonstrated greater than 90% viability, regardless of the exposure time.

Unfortunately, this study was unable to test infinite concentrations of chloramine-T at an infinite number of exposure times. Thus, it is not currently known if a 3-minute exposure is sufficient to kill *E coli*; nor is it known what the cytotoxic effect of a 5-minute exposure at 300 ppm would be. Further research that might show bactericidal effects at 3 minutes or acceptably low cytotoxic effects at 5 minutes would more strongly support the use of chloramine-T against *E coli*.

Based on the results of the present study, it appears that chloramine-T, at the concentrations used in this study, is bactericidal against *S aureus,* MRSA*,* VRE*,* and *E coli* and may be useful in reducing bacterial burden when these bacteria are colonizing wounds without harming fibroblasts. At this point, its efficacy for wounds colonized with *P aeruginosa* is questionable.

Although the findings of this study cannot be explicitly generalized to the in vivo treatment of wounds, it is interesting that, in view of the increase in microbial resistance to antibiotics, several investigators recommend short-term courses of antiseptic agents at appropriate dilutions and delivered appropriately to actively infected, chronic wounds.[6,11,12,21,33,34] In their review of several antiseptic studies, Drosou et al[21] concluded that all, except hydrogen peroxide, may provide clinically significant bactericidal effects while causing only transient cytotoxic effects from which the host cells recover. Moreover, in an electrochemical analysis, Hahn et al[35] found that when chloramine-T is dissolved in

water, the oxidative reaction quickly kills bacteria, fungi, and viruses even at low concentrations, and because of the irreversibility of the oxidative reaction, there is no possibility for the microorganisms to create resistance.

Some authors point out that the potential toxic effects of antiseptic agents likely occur because they are delivered to wounds for long periods at toxic concentrations.[2,21] However, when 5 common antiseptic agents were applied via soaked gauze at higher than safe concentrations to partial-thickness porcine wounds, none of the antiseptic agents had negative effects compared with saline controls.[23] Chloramine-T is chemically designed to release chlorine slowly into water, which may explain why a literature review found no reports of cytotoxicity or cross infection between patients who received wound treatments in whirlpools to which chloramine-T was added at concentrations of 100 and 200 ppm. As previously mentioned, the gradual release of chloramine-T relies on the chemical cleaving of chlorine-nitrogen bonds. This may explain why, in contrast to other antiseptics,[9,10] minimal cytotoxicity was found with exposure to chloramine-T at 100 and 200 ppm for any length of time and with brief (3-minute) exposures at 300 ppm.

Other antiseptic agents are incorporated into wound dressings containing vehicles such as cadexomer starch and hydrogels that enable ongoing release of the agent over several days, allowing the antiseptic to wield its short-acting effects for a longer period while maintaining a moist wound bed environment.[4,5] Thus, it has been suggested that the potential detrimental effects of antiseptic agents on wound healing may be related more to their method and duration of delivery than to their chemical effects.[2]

Implications for clinical use of chloramine-T

The present study has demonstrated that regardless of antiseptic concentration, water bath temperature, time of exposure, or presence of serum chloramine-T caused 95% to 100% growth reduction of 3 gram-positive bacteria: *S aureus*, MRSA, and VRE. The above results were replicated following exposure of a gram-negative bacterium, *E coli*, but not in the presence of serum. In the presence of serum, a concentration of 300 ppm was required to create a greater than 95% reduction in *E coli* growth.

When fibroblasts were exposed to chloramine-T at concentrations of 100 and 200 ppm, greater than 90% of fibroblasts were viable at 48 hours after exposure. At 300 ppm and a 3-minute exposure, almost 70% of fibroblasts were viable 48 hours later. In this study, chloramine-T was less toxic than other antiseptics previously tested in vitro.[9,10] Furthermore, a recent review of literature reported that, in the majority of in vivo clinical trials, antiseptic agents were not found to negatively influence wound healing.[21] Given that antibiotics increase the risk of bacterial resistance, create super infections, and promote nosocomial infections, the results of the present study seemingly make a case in favor of using topical antiseptics as alternatives for the treatment of wounds that have a high bacterial burden.[34]

Chloramine-T has had several decades of clinical use for preventing cross contamination between chronic wounds and burns of patients following whirlpool cleansing and irrigation. It contains 12.25% available chlorine and is distributed as a white powder that releases chlorine slowly when dissolved in water.[36] Although it is not available as a component of a wound dressing, it could be used as the

irrigating medium delivered by pulsed lavage with suction (PLWS). The advantage of using PLWS versus whirlpool as the chloramine-T delivery system is that PLWS can continuously deliver and simultaneously aspirate the antiseptic irrigant under controlled and safe positive and negative pressures, respectively. With the clinician holding the splash guard in contact with the wound to maintain the vacuum seal, the splash guard is moved over the wound surface for 3 to 10 minutes to remove slough and bacteria and to increase blood perfusion secondary to the effects of negative pressure. Maintaining constant contact of the splash guard with the wound bed would prevent antiseptic aerosolization, and pooling would be avoided by vacuum aspiration.

Summary

The present study has shown that in cell-friendly concentrations, chloramine-T can be effective against 3 gram-positive wound pathogens in vitro and that safe and effective concentration/exposure time combinations may exist for use against gram-negative organisms. Although further research-especially in vivo-is indicated, clinician reluctance to use chloramine-T, at least at the concentrations and exposure times discussed herein, may be unfounded.

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