An *In Vitro* Wound Healing Model Assessing Wound Healing Properties of Benzathonium Chloride Bioclay

Jeremy Kiripolsky, Dr. Paul Kostyniak, Dr. Patricia Masso-Welch, Department of Biotechnical and Clinical Laboratory Sciences, School of Medicine and Biomedical Sciences, University at Buffalo, 3435 Main Street, Buffalo, NY 14214

Abstract

Benzathonium chloride (BTC) is a quaternary ammonium compound (QAC) that is used as an antibacterial in several applications[1]. BTC can be complexed with laponite clay (Bioclay) particles, making it capable of being applied and retained topically[2]. Once complexed, BTC cannot be easily leached out of the clay by diffusion due to strong interactions with the clay surface. Bioclay has a wide range of potential applications for imparting antimicrobial activity in medical, industrial, and household applications. Bioclay has been previously shown to be effective against methicillin resistant *Staphylococcus aureus* (MRSA), and has been developed in cream form for topical application as an antibacterial cream[1]. This study investigates the hypothesis that BTC, when incorporated into a pharmaceutical grade colloid, can enhance the process of wound healing. This is done using an *in vitro* wound healing model utilizing a variety of cell lines. WI38 cells are normal diploid human fibroblasts. TM12 and TM12T are immortalized mammary epithelial cell lines;TM12T is a derivative of TM12 and has undergone epithelial to mesenchymal transition. The first study utilized the TM12 cells which were epithelial in appearance and when confluent looked cuboidal and tightly joined. The cells tended to maintain cell to cell connections after being scraped in order to induce the wound and as an attempt to prevent this flap of cells from falling back into the wound the well was washed with PBS before the different medias were added. In this study an induced "wound" on a confluent well is treated with media or media treated with either BTC or Bioclay. The wound width was measured after 24 hours to compare the width of the wounds between the control and treatment groups. A viability assay was run in parallel, to control for any effects of BTC compounds on total viable cell number. No effect was seen on wound healing at any of the concentrations tested, in either fibroblast, epithelial or EMT epithelial cell lines. Lack of effect was not due to loss of viability. Although no effect was seen of BTC compounds on these purified cell lines, *in vivo* wound healing may still be affected, due to the coordinated participation of several cell types, including granulocytes, macrophages, fibroblasts, and epithelial cells.

Introduction

Wound beds are complex environments in which dead tissue, exudate, and the bacterial bioburden interact in a complicated manner among themselves as well as the host. In these situations, bacteria tend to organize into biofilms, reducing their susceptibility to elimination as well as impairing healing[3]. The cleaning of the wound is one of the basics of standard care and help the healing of a chronic wound. Published literature shows that cleaning improves the wound environment and accelerates healing, In previous studies, BTC has proven to be affective against a number of bacteria including (but not limited to) *E. coli*, *H. influenzae*, *S. typhi*, *S. aureus*, and *S pneumoniae*[4]. Anecdotal evidence from topical use of Bioclay has indicated that BTC complexed with laponite can have a positive effect on wound healing. This is a fiirst attempt to understand the mechanism(s) by which this can occur.

Methods

Wound healing assays Cells were plated in 24 well plates at $5X10^4$ cells/well and grown to confluence. The confluent layer of cells in triplicate wells was then scratched from the top of the well down using a micropipette tip. Wells were rinsed with sterile PBS to remove detached cells. Cells were then fed with media containing 0, 1.25, 2.5, or 5 µg/ml BTC or BTC-clay. These concentrations were selected to be below concentrations previously used in a cream formulation (50 µg/ml), and based on literature that assessed the toxicity of BTC[4]. At 24 hours, images were taken using a phase contrast inverted microscope. Wound diameter was measured at two time points (0 and 24 hours) to assess % wound closure at 24h using the formula:

(Width at time zero) - (Width at 24h)) / (Width at time zero) X 100. A total of 3 plates were used in the wound healing assays of the TM12 and TM12T cells while there was only one plate used in the WI38 study

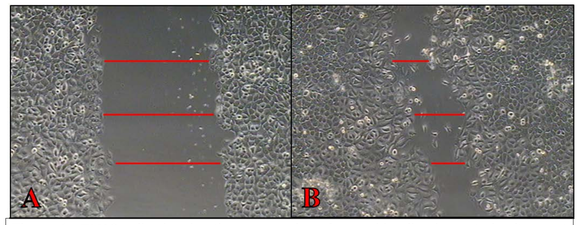

Figure 1. TM12 cells showing the progression of wound healing in control cultures at 0 (A) and 24 hours (B). The bars indicate measured distances used in calculating the percentage of the wound that has healed.

MTT Assay After photography of the wells, 200 µl of 5 mg/ml MTT was added to each well. After 4 hours, cells were photographed to show viability of cells in the culture. A colorimetric assay was then performed by harvesting cells quantitatively into isopropanol, and measuring the optical density of cells from each well at 595 nm.

Statistical Analysis All data Analysis was done using Sigma Plot 11.0. All of the groups were compared using one way ANOVAs with a significance level of 0.05. The bars in the graphs are standard error.

Results

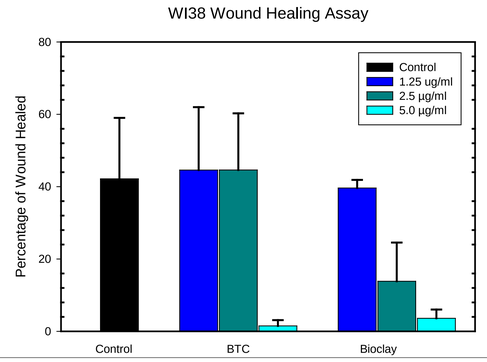

Figure 2. WI38 wound healing assay results after 24 hours. Although there is a decrease in wound healing seen in the graph, there is no statistical decrease in wound healing. There was 42.1% wound healing observed in the control group.

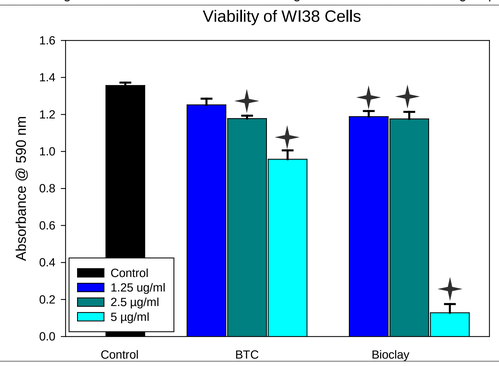

Figure 3. There is a statistical difference between the control group and all other groups which have the star symbol above them. These differences are statistically relevant with a P value equal to or less than 0.006. The decrease in cell viability explains the decrease in wound healing seen in the WI38 wound healing assay.

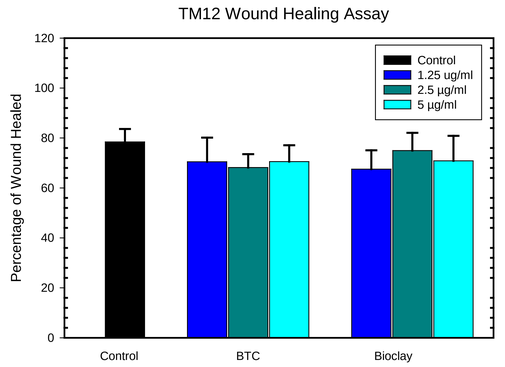

Figure 4. After 24 hours of incubation, the TM12 control group averaged 78.4% wound closure. The wound closure of the control group was not statistically different from any of the BTC or Bioclay variable groups (P=0.863).

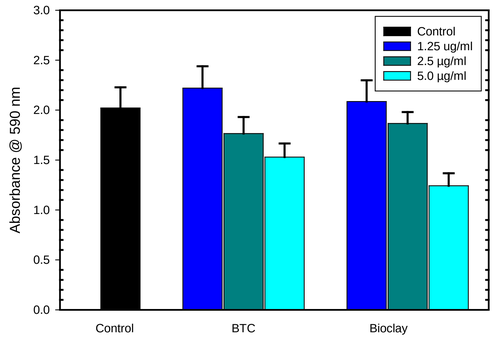

Figure 5. The TM12 MTT assay shows a slight decrease in cell viability as the concentration of either BTC or Bioclay increases however, there is no statistical (P < 0.05) difference between any of the groups with the control. There was a difference between the 1.25 µg/ml BTC group and the 5.0 µg/ml Bioclay group (P = 0.029).

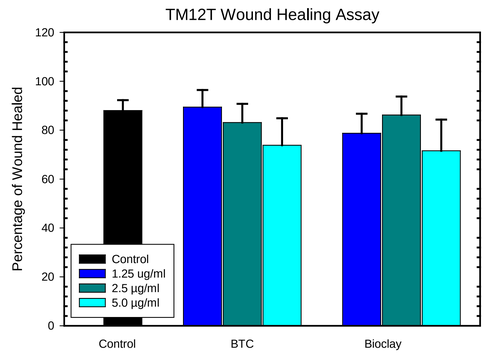

Figure 6. After 24 hours of incubation, the TM12T control group averaged 88.0% wound closure. The wound closure of the control group was not statistically different from any of the BTC or Bioclay variable groups (P=0.780).

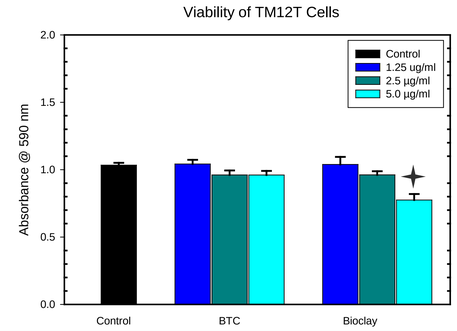

Figure 4. The TM12T MTT assay differs from the TM12 MTT assay in the fact that there is no decrease in cell viability (with a P < 0.05) except for the 5.0 µg/ml Bioclay group. The 5.0 µg/ml Bioclay group was different from all the other groups with a P value of at most 0.006. All of the other groups are statistically similar.

Discussion

- There was no enhancement of wound healing responses in either WI38,TM12 or TM12T cells by BTC or Bioclay.
- There was no difference in wound healing between the BTC and Bioclay groups for any of the cell lines used.
- The TM12 cell line was more sensitive to the BTC and Bioclay than the TM12T cell line.
- WI-38 fibroblast viability was more sensitive to Bioclay than BTC alone as seen in the viability assay.
  - At higher concentrations of BTC there was a slight loss of cell viability in TM12 cells, but TM12T cells were less sensitive to BTC which might be contributed to the cell's more tumorigenic properties.
  - The use of the TM12 and TM12T cells as an *in vitro* wound healing model demonstrated that BTC and Bioclay do not inhibit wound healing in concentrations as high as 5.0 µg/ml. There was also minimal effects on cytotoxicity at these levels.
- There are limitations to using a single cell *in vitro* model , such as the lack of cell to cell interactions between the many different cells in the wound bed, that will require the use of an *in vivo* model to observe.
  - The lack of an *in vitro* effect may not predict an *in vivo* effect. The *in vitro* studies do not mimic the *in vivo* environment in which multiple cell types (granulocytes, macrophages, fibroblasts, and epithelial cells) participate. An *in vivo* wound healing assays will be useful to test the effects of Bioclay on myriad cell types in the physiological wound bed.
- This study suggests that the mechanism of any BTC induced increase in wound healing *in vivo* is not likely a result of a direct effect on cellular proliferation, but rather, through its antimicrobial effect of decreasing microbial proliferation within the wound bed.

References

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