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13. SUPPLEMENTARY NOTES

14. ABSTRACT This one year project investigated (in vitro) the ability of microsphere-based chitosan-calcium sulfate composites to locally deliver growth factor and antibiotics to heal bone and prevent infection in traumatic musculoskeletal injuries. Antibiotic, vancomycin (vanc) was loaded into calcium sulfate (CaS) and microspheres loaded with either vanc or bone morphogenetic protein 2 (BMP-2). In vitro release was evaluated over 6 weeks and biological activity and interactions of released factors were evaluated in cultures. Composites released vanc at levels (>40µg/ml) that killed *S. aureus* for 18 days. BMP-2 release from microspheres was reduced to ~1ng/ml/day when combined with CaS as compared to plain microspheres (~9ng/ml/day for days 6-10 elution). This may be due to high affinity of BMP for calcium and hence may serve to retain BMP in wound site. There was no overt toxicity with levels of vanc (<3600µg/ml) and BMP-2 released, but the presence of vanc suppressed (~20-50%) BMP-2 induced alkaline phosphatase expression in cells. Additional work will need to determine retention of BMP by composite and long term effects of vancomycin on BMP-2 stimulated osteogenesis.

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INTRODUCTION:

The current conflicts in Iraq and Afghanistan are resulting in complex and traumatic musculoskeletal injuries, especially in the extremities, to military personnel that are difficult to treat because of comminution of the bone, infection and severely damaged vasculature. For these injuries, it would be ideal to have a graft material that is effective at healing and regenerating bone and reducing or preventing infectious complications through local delivery of therapeutic agents. The long-term hypothesis of this research is that a microsphere-based chitosan-calcium sulfate composite is able to locally deliver therapeutic agents to prevent wound infection and to stimulate bone healing and regeneration. The purpose of this one year research project was to examine, in vitro, the ability of a microsphere-based composite scaffold to temporally deliver two therapeutic agents; an antibiotic (vancomycin) for 2-4 weeks for treating/preventing infection and a 7-10 day release of bone morphogenetic protein (BMP-2) to stimulate bone healing and regeneration. These times match current clinical strategies for treating infection and stimulating bone healing. The composite scaffold material is composed of chitosan, a natural polysaccharide, and calcium sulfate, a bone like mineral. Both chitosan and calcium sulfate (CaS) are biocompatible, degradable, and are already used in clinical applications to help stop bleeding and treat bone injuries. The scope of work was to; 1] Measure and evaluate the release kinetics of the antibiotic and growth factor and 2] determine the biological activity and potential interactions between the antibiotic and BMP-2 on cultured bacteria and bone cells.

BODY:

This project addressed two specific goals with the following milestones;

Goal 1: To optimize composite scaffold to provide a sustained 2-4 week release of vancomycin and a 7-10 day release of BMP-2 beginning one week after implantation.

This goal involved the mixing of chitosan microspheres, containing vancomycin or BMP-2 (via swelling adsorption) with a fast resorbing calcium sulfate containing vancomycin. Temporal release of therapeutic agents from composites was evaluated over six weeks in simulated physiological solutions. Released vancomycin was measured by standard immunoflourescence, and BMP-2 by ELISA. The ratio of vancomycin and BMP-2 containing microspheres was varied in composite scaffolds to determine effects on release. The milestones for goal 1 were:

Milestone 1: Prepare of chitosan microsphere scaffolds infused with calcium sulfate.

Milestone 2: Determine the six week release of the antibiotic and growth factor from composites in vitro.

Goal 2: To determine the biological activity and potential interactions between the antibiotic and BMP-2 on bacteria and bone cells in culture.

This goal evaluated the biological activity of released agents in eluents from elution studies. Antibacterial activity was measured in turbidity and colony forming unit assays using *P. aeruginosa* and *S. aureus* to determined inhibition and killing of bacteria respectively. Bioactivity of released BMP-2 was determined using the W-20-17 cell line since it shows a dose-

dependent increase in ALP activity in response to BMP-2. Toxicity of released therapeutic agents and composite degradation products were determined using cultured bone cells. Interactions between vancomycin and BMP-2 on bone cell viability and activity to stimulate ALP were also evaluated in bone cell cultures. The milestones for this goal were;

Milestone 3: Determine the ability of released vancomycin to inhibit growth of two model bacteria associated with military orthopeadic injuries.

Milestone 4: Measure viability of cultured bone cells exposed to solutions containing released drugs. Saos-2, bone cells will be incubated with elution samples at 37C for 24 hours. The viability will then be checked via a mitochondrial assay (CellTiter® AQ, Promega, WI).

Milestone 5: Measure the biological activity of released BMP-2 in the presence of released vancomycin on bone cells in culture. W-20-17 cells (ATCC# CRL-2623) will be incubated with elution samples for 24-48 hours at 37C. The cells will then be lysed and cellular alkaline phosphatase (ALP) activity will be determined spectrophotometrically using the p-nitrophenol assay. A BCA total protein assay will also be used to evaluate the lysates in order to normalize the ALP data.

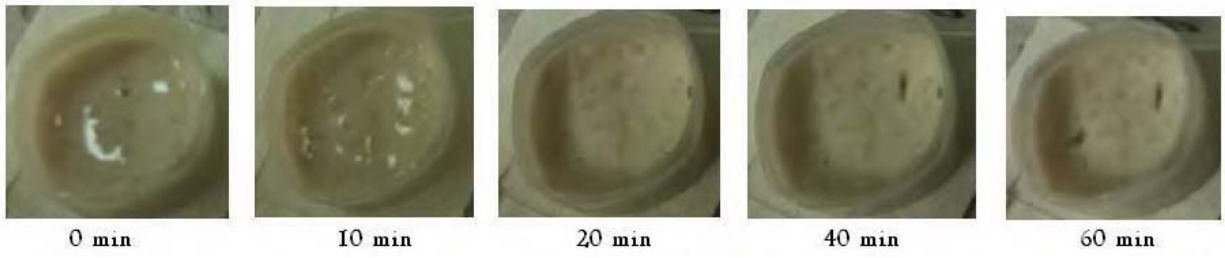
Results:

Milestone 1: Preparation of chitosan microsphere scaffolds infused with calcium sulfate.
Goal of this milestone was to make a chitosan-microsphere scaffold with calcium sulfate-vancomycin slurry and have it set within 30-60 minutes in order that the composite material may be prepared in the operating room prior to surgery.

Set time of hydrated composite beads (calcium phosphate and chitosan) in calcium sulfate (CaS) was performed. The amounts of solution for rehydrating 100mg of beads and amount of solution used for mixing CaS are varied as shown in Table 1. Beads (100mg) were hydrated for 15 min at room temperature (RT). CaS (600mg) was mixed with dI H_2O. The hydrated beads were then added to the CaS, mixed thoroughly and observed over 60 min for set time. Beads hydrated in 200 and 250 µL did not absorb the solution fully. 100 µL was not able to fully contact all of the beads. Beads loaded with 150 µL dI H_2O performed the best in the set test, hardening the fastest based on when added to CaS dissolved in 220 µL. Scaffolds with CaS dissolved in 240 µL dI H_2O took over 60 min to fully harden. CaS dissolved in 160 µL was too viscous to mix well with the beads. Scaffolds prepared with beads hydrated with 150 µL and CaS dissolved in 180 µL appeared to harden fully by 60 min [Figure 1]. Samples were visually assessed as well as poked with a metal spatula. Samples which could not be easily indented were considered hardened. Results were confirmed using 33.3 ng/ml BMP-2 in PBS for microspheres and 3.33ug/ml vancomycin in dI H_2O.

Table 1: Ratios of fluids to 100 mg chitosan microspheres and 600 mg calcium sulfate used to determine composite set times.

Beads Hydrated in PBS (µL)	CaS dissolved in dI H_2O (µL)				
100	240	220			
150	240	220	200	180	160
175	240	220			
200	240				
250	240				



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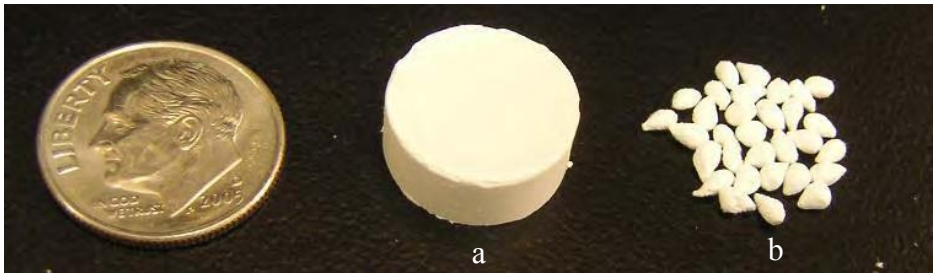

B

Figure 1: A] Photograph series of Scaffold set-time from mixing beads hydrated in 150µL PBS and CaS dissolved in 180µL dIH_2O and B] of set (a) composite scaffold and of (b) unhydrated chitosan microspheres

Milestone 2: Determination of the six week release of the antibiotic and growth factor from composites in vitro.

The goal of this objective is to achieve release of vancomycin from the composites for 2-4 weeks at greater than 8-16µg/mL (a minimum inhibitory concentration for antibiotic resistant strains of *S. aureus*[1], and a sustained 7-10 day release of greater than 10ng BMP-2/mL.

Microspheres were made to contain BMP-2 or vancomycin via swelling. Microspheres mixed in different ratios were combined with CaS-vancomycin slurry in silicone molds and allowed to set for 60 min (Table 2). Control scaffolds containing only vancomycin microspheres, only BMP-2 microspheres, or no therapeutic agent (i.e. no vancomycin in either the microspheres or calcium sulfate and no BMP-2 containing microspheres) were used as controls. Elution of BMP-2 and

antibiotic from composite scaffolds was carred out in culture medium supplemented with 10% serum at 37^{o}C to simulate extracellular fluid environment. Eluates were collected at designated time points over 6 weeks (42 days). Elutates were analyzed for BMP-2 release by ELISA (ELISA development kit (detection limit=62.5 pg/mL, Peprotech, Rocky Hill, NJ) and for vancomycin by Florescence Polarization Immunoassay (TDxFLx, Abbott Laboratories, Abbott Park, IL) are shown in Figures 3-5.

Table 2: Key to composites and relative loading of vancomycin and BMP-2

Group	Amount of vancomyin in the CaS shell (mg) (2% wt vancomycin)	Relative mass ratio of BMP-2 containing beads to vancomycin containing beads within CaS shell	Amount of BMP-2 (µg) in chitosan-CaP composite based on relative ratio of beads (46±8µg BMP-2/g microsphere)	Amount of vancomycin (mg) in the chitosan-CaP composite based on relative ratio of beads (mg) (9.3±2.2mg vancomycin/g microsphere)
H	15.6	0:4	0	1
I	15.6	4:0	5	0
J	15.6	1:3	1.25	0.75
K	15.6	2:2	2.5	0.5
L	15.6	3:1	3.75	0.25
M*	15.6	--	0	0
N*	0	--	0	0

* controls

Figure 3 shows elution over the first 11 days, and Figure 4 shows elution from day 14-42. Note control sample N is not shown on these figures because the values were zero. Two way ANOVA indicated that there were significant differences over time ($p< 0.001$) and between groups ($p< 0.007$) but there was a significant interaction between time and groups ($p< 0.001$). In general, post hoc analyses using Student-Newman-Keuls (SNK) at the $p=0.05$ level of significance showed that the amount of vancomycin released decreased significantly at each measurement time point for all groups until day 9. After day 9 vancomycin release continued to decrease for all groups but was not statistically significant at between all time points. Comparing groups, statistical differences were consistently identified between H (0% BMP-2: 100% vancomycin loaded microspheres) and I (100% BMP: 0% vancomycin loaded beads). However, differences were not consistent between other groups which accounts of the significant interaction detected between groups and time factors.

Vancomycin release above 8µg/mL was achieved for over two weeks which was within our target range for all groups except for N (negative control group). However, there was no difference in levels or time sequence of release for composites that contained vancomycin in both microsphere and CaS components. This was attributed to the low amount of vancomycin loaded into the microspheres relative to the large amount of vancomycin loaded into the CaS component (e.g. 100mg vancomycin microspheres contained only 0.9mg vancomycin compared to the 15.6 mg vancomycin loaded in the CaS). The release profile of vancomycin shown here is

similar to that of other studies, where there is an initial burst release followed by lower, yet sustained release for up to 33 days[2,3].

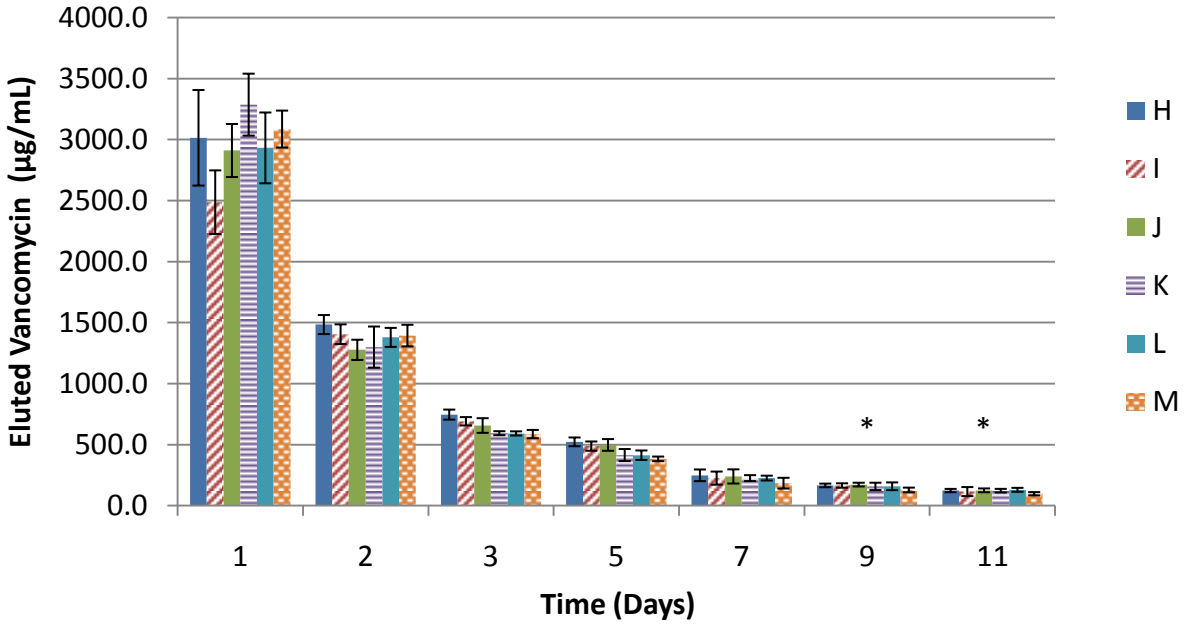

Figure 2: Vancomycin Elution from CaS-chitosan composites (n=5). Day 1 - Day 11: Levels of vancomycin release ranged from greater than 2500 µg/mL at day 1 to greater than the 95 µg/mL at day 11. Values exceed target value of 8-16µg/mL range. Symbols indicate no statistical difference at p=0.05.

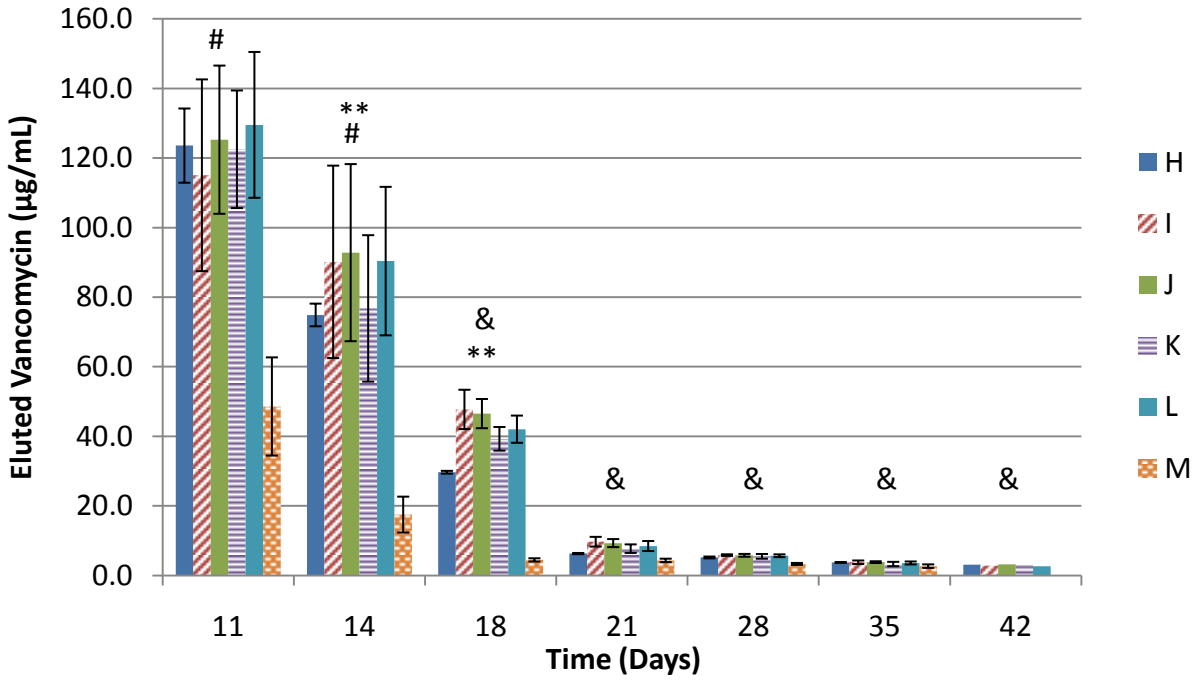

Figure 3: Vancomycin Elution from CaS-chitosan composites (n=5). Day 14 - Day 42: Levels of vancomycin release ranged from >48 µg/mL at day 14 to >17 µg/mL at day 18. Values exceed target range of 8-16µg/mL for the specified time frame of up to 2 weeks. Note at day 21, composites I, J and L continued to release levels of vancomycin above target range thus exceeding the minimum specified time frame for release. Levels of vancomycin release were <8 µg/mL for remaining time points measured. Symbols indicate no statistical difference at p=0.05.

BMP-2 release from composite scaffolds (Figure 4) did not achieve target release levels of 10ng/ml for 7-10 day time frame. It was speculated that the BMP-2 was being retained by the calcium in the composites. Two factor ANOVA indicated that there were significant different in BMP-2 release over time and between groups ($p<0.001$) and there was a significant interaction between time and group factors ($p<0.001$). In general using the SNK post hoc analyses for groups, there is a trend for BMP-2 release to be statistically proportional to amount of BMP-2 loaded into composites (Figure 4), but differences were not consistent between groups at each evaluation time point.

To investigate the possibility of BMP-2 retention by calcium sulfate, chitosan microspheres were loaded with BMP-2 and eluted for 6 weeks in culture medium. To check the effects of sampling intervals, two groups of beads were tested; in one group eluates were collected daily for six weeks and the second group eluates were collected at intervals. Figure 5 shows the cumulative release of BMP-2 from each group. Statistical analyses with two factor ANOVA and SNK at showed that there were no differences in levels released between groups for days 1 through 7 ($p>0.05$). In this time frame, release of BMP-2 was greatest for both groups and for the interval group, intervals were short ranging between 1 and 2 days. Hence differences would not be expected. However, after day 9, the daily sampled group showed consistently greater levels of release as compared to the interval group ($p<0.05$). This indicates that there is some loss of BMP-2 due to degradation in the solution during the 3-7 day intervals. Thus more BMP-2 is

detectable in the daily sampled group as compared to the interval sampled group. This result is significant in that typical interval sampling is likely to underestimate the BMP-2 release levels. Also the BMP-2 released from microspheres alone as compared to the CaS-microsphere composites was greater and sustained at between 9.5-7.2 ng/ml at days 6-10 which is close to our target value. Furthermore, it was observed that BMP-2 release was sustained for the entire 6 week period from the microspheres alone with 1.0 - 2.2 ng/ml being continuously measured over days 36-42. This elution profile is similar to that of Reves, et al, where BMP-2 elution from lyophilized chitosan microspheres was sustained for 26 days[4]. Based on these results, it is concluded that BMP-2 is being released from the microspheres and that combining the microspheres with CaS slows release and potentially helps to retain the BMP-2 locally. This later aspect may be very important to helping maintain BMP-2 in the wound site as compared to being lost with turn-over of wound fluids.

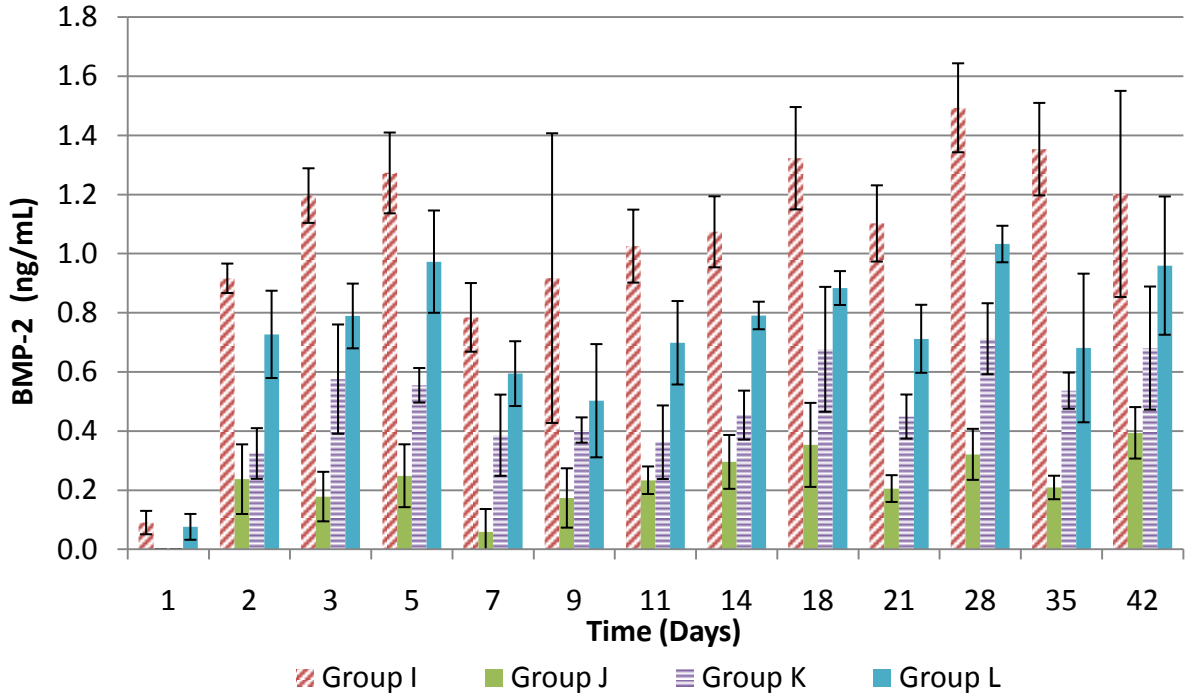

Figure 4: BMP-2 Elution from Calcium sulfate-chitosan composites (n=5). Group I contained the most BMP-2 (5 µg/pellet) > Group L(3.75 µg/pellet) > Group K (2.5 µg/pellet) > Group J (1.25 µg/pellet).

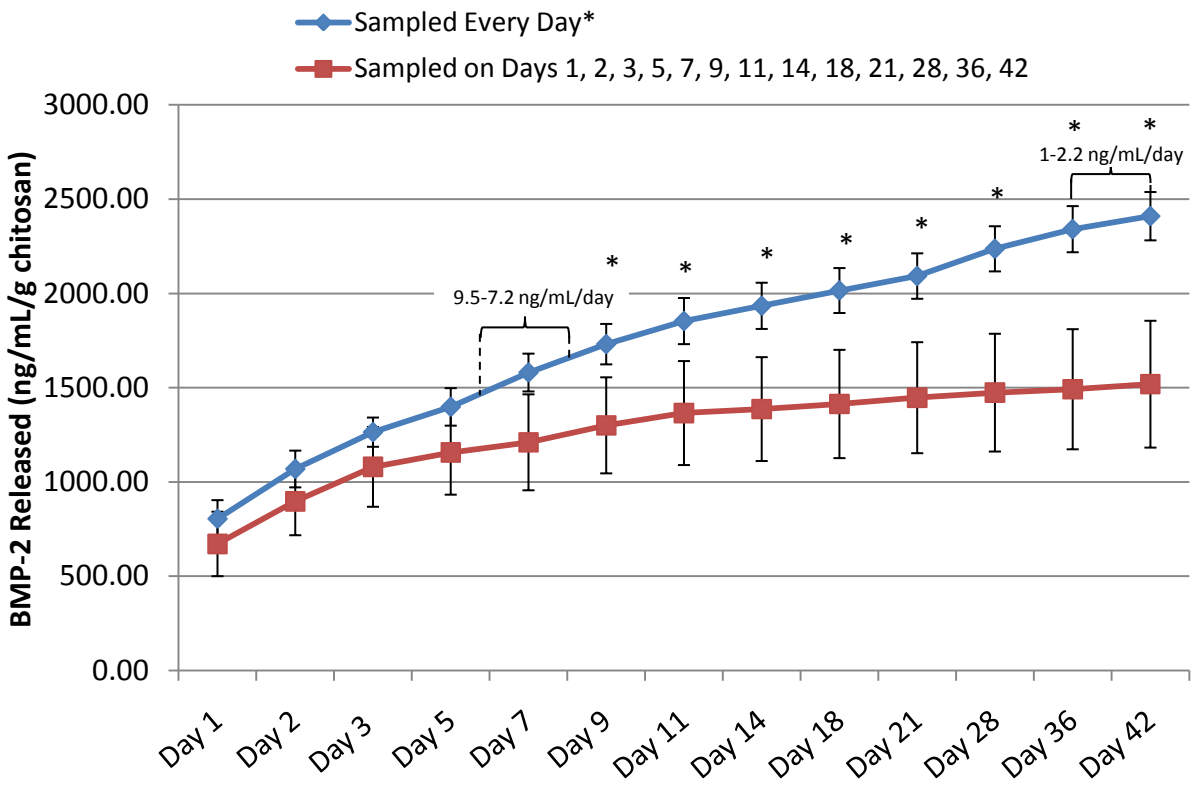

Figure 5: BMP-2 release from chitosan microspheres in medium +1% Bovine Serum Albumin (BSA), a component used to reduce non-specific adsorption of BMP-2. For daily sampled test group, samples on Days 30, 35 and 38 were lost and are not included in data. There was a significant difference in the amount of BMP-2 measured between daily sampled and interval sampled groups after day 5. By the end of the experiment, 40% less BMP-2 had been measured for the interval group as compared to the daily sampled group.

Milestone 3: Determine the ability of released vancomycin to inhibit growth of two model bacteria associated with military orthopeadic injuries.

Eluents collected over 6 weeks from test constructs in Table 2 were tested for ability to inhibit the growth and kill model bacteria, *S. aureus (*Cowan I strain) and *P.aeruginosa* (strain ATCC 27317), associated with infected orthopaedic wounds. The ability of the eluents to inhibit bacterial growth was determined in turbidity tests. The ability to kill bacteria was based on the number of colony forming units from surviving bacteria in turbidity cultures. Tables 3 and 4 shows the results of antibacterial tests to the *S. aureus (*Cowan I strain). The amount of vancomycin in the released eluents was 99.9% effective against *S. aureus* through day 18. Figure 6 shows representative plates with greater than 99.9% reduction in colony forming units.

Table 3: Turbidity results of vancomycin against *S. aureus* (n=5). (-) indicates no growth, (+) indicates growth and (±) indicates growth in some sample and no growth in other samples. This is due to variation in drug release between individual test samples at low levels approximating the minimal inhibitory concentrations. All groups with vancomycin showed inhibition of bacterial growth for 18 days. Groups I, J, K and L which contained vancomycin beads also showed strong inhibition of bacterial growth for up to 3 weeks for most samples. All (+) groups were found to be statistically different from (- and ±) groups ($p<0.05$).

	Days												
Group	**1**	**2**	**3**	**5**	**7**	**8**	**11**	**14**	**18**	**21**	**28**	**35**	**42**
H	-	-	-	-	-	-	-	-	-	+	+	+	+
I	-	-	-	-	-	-	-	-	-	±	+	+	+
J	-	-	-	-	-	-	-	-	-	±	+	+	+
K	-	-	-	-	-	-	-	-	-	±	+	+	+
L	-	-	-	-	-	-	-	-	-	±	+	+	+
M	-	-	-	-	-	-	-	-	-	+	+	+	+
N	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4: Ability of eluents to kill *S. aureus* (n=5). (√) indicates that the eluents reduced the number of colony forming units from surviving bacteria in turbidity tests by 99.9%. Data confirm that the amounts of antibiotic released from vancomycin containing composites were effective at killing bacteria for up to 18 days. All (√) groups were found to be statistically different from unmarked or (*) groups ($p<0.05$).

	Days												
Group	**1**	**2**	**3**	**5**	**7**	**8**	**11**	**14**	**18**	**21**	**28**	**35**	**42**
H	√	√	√	√	√	√	√	√	√				
I	√	√	√	√	√	√	√	√	√	*			
J	√	√	√	√	√	√	√	√	√	*			
K	√	√	√	√	√	√	√	√	√	*			
L	√	√	√	√	√	√	√	√	√	*			
M	√	√	√	√	√	√	√	√	√				
N													

*partial killing

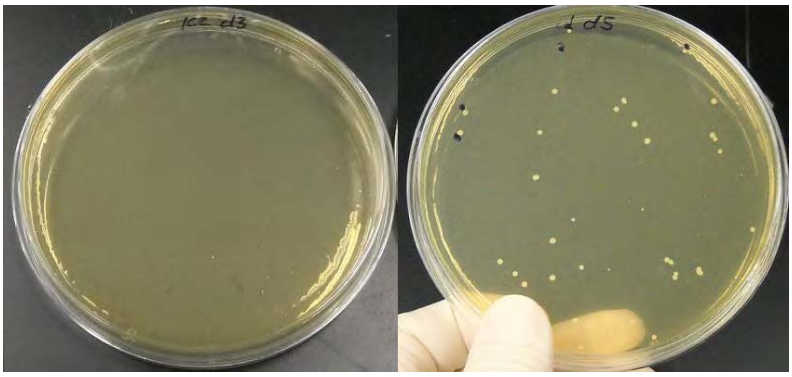

Figure 6: Representative images of reduction in colony forming units of surviving bacteria from turbidity studies of test eluents. Image on left shows no colony forming units from a group K sample, day 3 elutent. Image on right shows greater than 99.9% reduction in colony forming units from group L sample day 5.

Table 5 shows data for turbidity results against *P.aeruginosa* (strain ATCC 27317). Vancomycin is effective against gram positive bacteria and therefore no bacterial growth inhibition was noted in turbidity assays for the gram negative bacterial species, *P.aeruginosa* so, no bactericidal (plating for surviving colony forming units) assays were performed.

Table 5: Turbidity results of vancomycin against *P.aeruginosa* (n=5). (+) indicates bacteria growth. No group showed inhibition of bacterial growth.

	Days												
Group	**1**	**2**	**3**	**5**	**7**	**8**	**11**	**14**	**18**	**21**	**28**	**35**	**42**
H	+	+	+	+	+	+	+	+	+	+	+	+	+
I	+	+	+	+	+	+	+	+	+	+	+	+	+
J	+	+	+	+	+	+	+	+	+	+	+	+	+
K	+	+	+	+	+	+	+	+	+	+	+	+	+
L	+	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+	+
N	+	+	+	+	+	+	+	+	+	+	+	+	+

Vancomycin is most effective against gram positive bacteria; therefore, no bacterial growth inhibition was noted in turbidity assays for the gram negative bacterial species, *P.aeruginosa*. Thus the bactericidal assays (plating for surviving colony forming units) assays were not performed. A broad spectrum antibiotic effective agains gram negative species such as Amikacin would need to be considered in wounds containing such bacteria.

These results demonstrate the potential for composites to provide sustained release of antibiotic agents at levels that are effective at killing and inhibiting growth of gram positive bacteria associated with infected orthopeadic wounds. In a situation of a sever orthopeadic wound, there may be multiple species of bacteria present, in such cases, more than one antibiotic might be necessary to clear the infection. Two or more antibiotics could be loaded into the scaffold, which

is a common clinical practice[3], however, it would be necessary to first test the reaction of the antibiotic together to ensure effectiveness and non-toxic results.

Milestone 4: Measure the viability of cultured bone cells exposed to solutions containing released drugs. Saos-2, bone cells will be incubated with elution samples at 37C for 24 hours. The viability will then be checked via a mitochondrial assay (CellTiter® AQ, Promega, WI).

Statistically, all of the test eluates from composites samples that contained no antibiotic or growth factor significantly reduced (17-50%) the viability of Saos-2 cells as compared to cell growth in medium alone (Figure 7) ($p<0.05$). The decrease in cell viability is attributed to the high levels of calcium and/or sulfate ions released from the composites which may alter the osmolarity of the medium and or interfere with the ability of the cells to remain attached to culture substrates. Both changes in osmolarity and loss of substrate attachment for substrate dependent cells may contribute to an impairment of metabolic activity and hence reduction in viability. These changes are an artifact of the initial elution set-up in which relatively small sample surface area (7.8 cm^2) to volume ratio (2 ml)is used. Because of the reduction in viability of the cells due to the high concentrations of calcium / sulfate ion degradation products, viability of the cells to composites that contained antibiotics and growth factor were not tested.

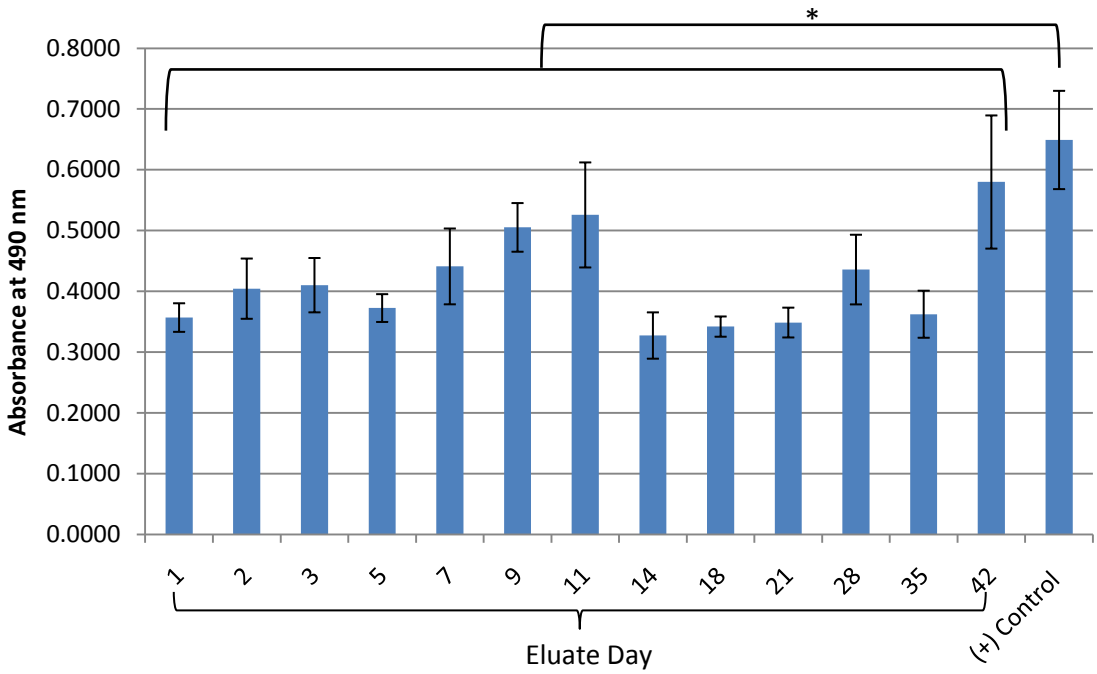

Figure 7: Saos-2 cell viability in the presence of eluates from composite pellets without antibiotics or growth factor (n=5). Saos-2 cells were exposed to 200µL of eluate or medium for 24 hours. All eluates significantly reduced cell viability as compared to control medium due to the high levels of calcium and or sulfate ions released from the composites. Asterisk (*) indicates statistical significance at $p<0.05$ with SNK post hoc tests.

Milestone 5: Measure the biological activity of released BMP-2 in the presence of released vancomycin on bone cells in culture. W-20-17 cells (ATCC# CRL-2623) will be incubated with

elution samples for 24-48 hours at 37°C. The cells will then be lysed and cellular alkaline phosphatase (ALP) activity will be determined spectrophotometrically using the p-nitrophenol assay.

To determine if there was any effect of the released antibiotic on the bioactivity of BMP-2, mock eluate solutions of BMP-2 and vancomycin were made (Table 6). The mock solutions were exposed to the W-20-17 murine cell line and evaluated for effects on viability and expression of ALP by the cells. The range of BMP-2 tested was selected in part to cover the range of values measured in the eluates, as well as, to values known to stimulate ALP expression in W-20-17 cells. Vancomycin levels were selected to cover the range of values measured in test eluates. Results of the effects of mock solutions on cell viability are shown in Figure 8 and on ALP expression in Figure 9. Statistical analyses with two way ANOVA indicated that there were no significant differences between BMP-2 dose level ($p=0.578$) or interactions between the two groups ($p=0.984$) but some of the mock solutions that contained vancomycin caused decreases in cell viability ($p=0.04$ to $p<0.001$). The decrease in viability did not however follow a trend, and there was only a slight decrease in viability ($P=0.04$) in cells exposed to 3600 µg/mL vancomycin compared to cells exposed to no vancomycin. Therefore, it may be assumed that no overt cytotoxicity would be associated with the varying levels of vancomycin and BMP-2 that were measured in original eluate solutions.

Table 6: Test solutions for cell viability and BMP-2/vancomycin interaction studies. Test solutions were made in DMEM medium.

Vancomycin Concentration (µg/mL)	BMP-2 Concentration (ng/mL)
3600	1000, 500, 100, 50, 10, 0
1800	1000, 500, 100, 50, 10, 0
450	1000, 500, 100, 50, 10, 0
112.5	1000, 500, 100, 50, 10, 0
0	1000, 500, 100, 50, 10, 0

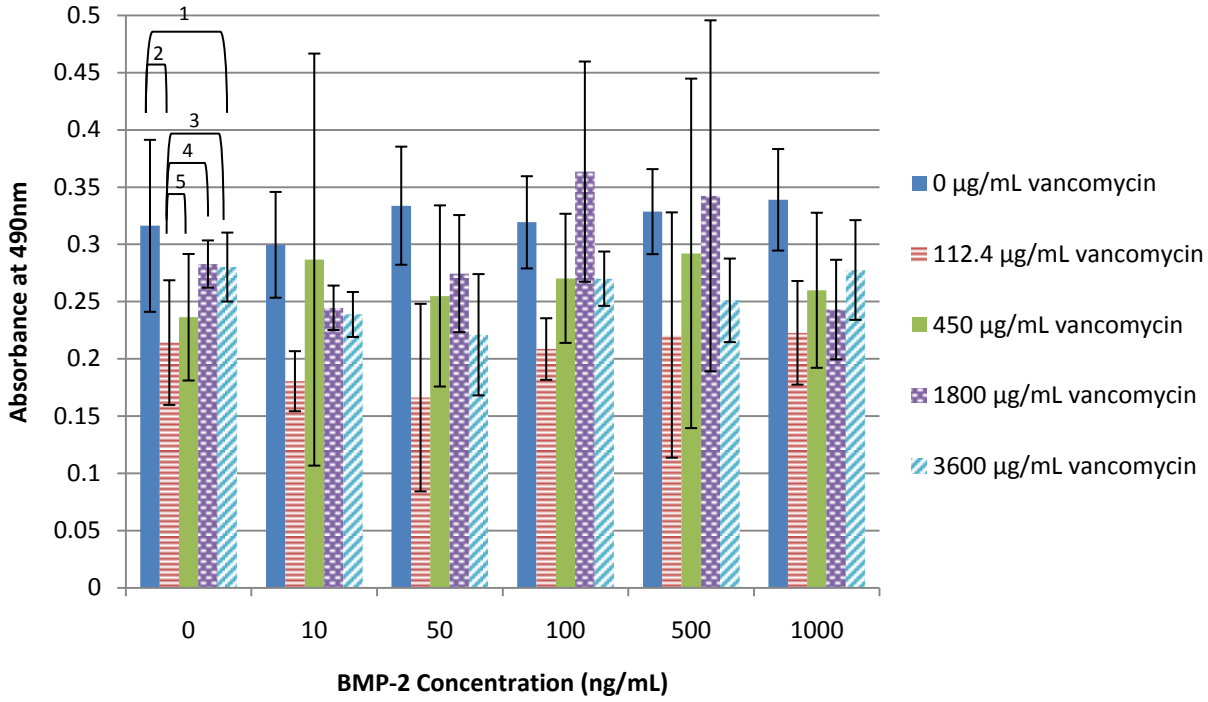

Figure 8: W-20-17 cell viability in the presence of BMP-2 and vancomycin (n=6). W-20-17 cells were grown for 24hrs in 200uL of pharmaceutical agent or control medium. Numbers indicate statistical differences, 1) P=0.04, 2) P<0.001, 3)P =0.029, 4) P=0.003, 5) P=0.026. Statistical differences do not follow a trend indicating that the level of vancomycin does not affect cell number.

While there was not an overt cytotoxic effect of the mock eluates on the cells, all mock eluates that contained vancomycin did result in a suppression of ALP by the W-20-17 murine cells as compared to BMP-2 mock solutions that did not contain any vancomycin (Figure 9). The inhibition of ALP expression was proportional to the amount of vancomycin in the mock solutions with highest concentration of vancomycin tested (3600µg/ml) showing the most inhibition and the lowest concentration (112.5µg/ml) showing the least. Two way ANOVA indicated that there were significant differences between BMP-2 dose level ($p < 0.001$) and between vancomycin dose level($p < 0.007$) as well as significant interaction between the two ($p < 0.001$). At 50ng/mL-1000ng/mL BMP-2 cells exposed to 112.5µg/mL vancomycin were statistically different from cells exposed to 1800 and 3600 µg/mL (SNK, $p<0.05$). As BMP-2 levels increase, more statistical differences were seen between the groups. This indicates that released vancomycin levels may interfere with BMP-2 stimulated osteogenesis. It is noted that the highest levels of vancomycin released are in the early initial days when inhibitory effects on BMP-2 would be maximum, but because of the lack of osteoprogenitor cells in the wound site this initial inhibitory effect may not be critical. Nevertheless the long term effects of moderate to low levels (<1800µg/ml) of vancomycin on BMP-2 stimulated osteogenesis will need to be determined.

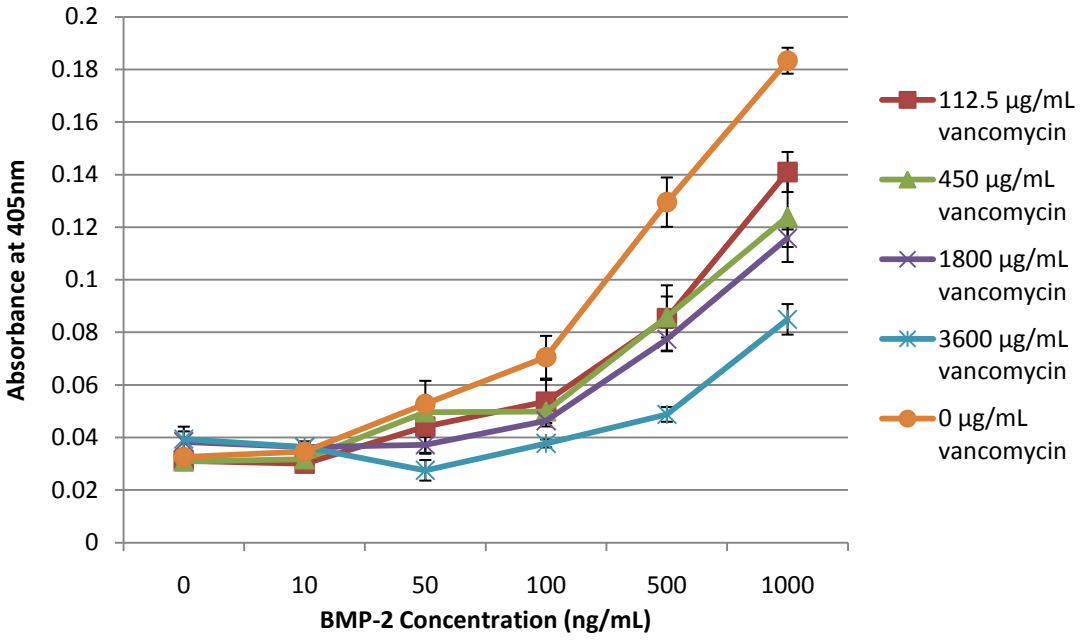

Figure 9: ALP production by W-20-17 cells in response to varying levels of BMP-2 in the presence of vancomycin (n=6). W-20-17 cells were grown for 24hrs in 200µL of pharmaceutical agent or control medium.

Additional work is continuing on these composites. Cell culture tests are currently underway to determine if changes in viability of the cells as exposed to the mock vancomycin and BMP-2 elutes are due toxicity of the vancomycin or due to any induction of cell apoptosis. The bioactivity of BMP-2 released from microspheres will also be evaluated in cell cultures. However because of the difference in medium used to perform the elution study and the medium requirements for the W-20-17 cells, an alternative method for assessing bioactivity of the released BMP-2 is being explored[4]. The method will evaluate the proliferation and ALP expression of Saos-2 cells over a 1-6 day culture period. Finally, since the amount of BMP-2 released from both the composites and the microspheres is still much lower than initial loading levels, an acid extraction method is being developed to determine how much BMP-2 remains. These results will be included in future publications planned for this work.

KEY RESEARCH ACCOMPLISHMENTS:

- Chitosan-calcium sulfate composite pellets can be loaded and assembled within 1hour at point-of-care for use in local delivery of antibiotics and growth factors.
- The composite pellets are capable of delivering levels of antibiotics that are able to kill and inhibit growth of model bacterial in vitro. This ability is dependent on the species of bacteria and the type of antibiotic loaded into the composite.
- While in vitro cell viability is not affected by levels of vancomycin released from composites, there may be a potential inhibitory effect on BMP-2 induced osteogenesis.

- BMP-2 was continuously released from chitosan microspheres over a 6 week period and release was decreased by combining microspheres with calcium sulfate.
- The amount of BMP-2 measured in eluates from chitosan-calcium sulfate composites were at sub-therapeutic levels but because BMP-2 has a high affinity for calcium materials this may potentially help retain BMP-2 in the local wound site.
- When sampled at daily intervals, higher levels of BMP-2 released in to physiological solutions at 37°C were measured as compared to multiday interval samples. Multiple daily interval sampling underestimates release levels of BMP-2 due most likely to degradation of the BMP-2 molecule over time.

REPORTABLE OUTCOMES:

- Abstract and Presentation:
    - H. Doty, W.O. Haggard, H.S. Courtney, J.D. Bumgardner, "Effectiveness of a Dual Drug Delivery Calcium Sulfate, Chitosan-Calcium Phosphate Bone Scaffold," Transactions of Society for Biomaterials 2010 Annual Meeting and Exposition, Seattle, WA, April, 2010

CONCLUSION:

To address the current clinical problem of treating bacterial infections and stimulating fracture healing in complex musculoskeletal injuries, a dual antibiotic and growth factor delivery and bone scaffold was made and evaluated. The first goal of this study was to create a fast setting scaffold which a surgeon would be able to prepare at the bedside of a wounded patient. Calcium Sulfate and chitosan microspheres were combined with vancomycin, a therapeutic agent used for the treatment of gram positive bacteria, and BMP-2 a potent growth factor which stimulates osteogenesis, in specific proportions to yield a fully set and ready to use scaffold in 60 minutes. Thus a product such as this could be used at the bedside and prepared directly before surgery, allowing the surgeon to tailor the components of the scaffold.

The scaffolds were eluted over 6 weeks and analyzed for the effective bacterial abatement/killing and to determine the osteogenic factor. The elution of the antibiotic proved effective in killing *s. aureus* for 18 days, which falls within the range of 2-4 weeks of bacterial inhibition as described in the 2nd milestone. The limitation of this finding is that vancomycin is only effective against gram positive bacterial, thus wounds with gram negative bacteria, or resistant strains would need different variations of antibiotics. In such cases the scaffold would need to be loaded with the antibiotic of choice, and potentially more than one antibiotic, without toxicity issues to the patient.

The osteogenic factor could not directly be determined from the eluted samples due to the high amounts of calcium and sulfate in the eluates, however, mock eluates were made with similar concentrations of eluted factors and used to evaluate potential interactions in vitro studies. It was determined that the level of BMP-2 eluted was not at effective level to stimulate a murine BMP-2-responsive cell line. It is hypothesized that BMP-2 is being retained by the scaffold due to the affinity of BMP-2 for calcium, thus, minimal but prolonged release was observed. Even though this release did not meet the goal to sustain a 7-10 day release of a biologically effective level of

BMP-2, there was a delayed release which would be important in a healing wound to allow time for the osteoprogenitors to migrate to the area.

To overcome the retention of BMP-2 in the scaffold, the 6 week assay was performed with BMP-2-loaded chitosan microspheres without calcium sulfate. The amount of BMP-2 eluted from the chitosan microspheres was over 20 times greater than the BMP-2 eluted from the composite calcium sulfate scaffolds. It is suspected that the BMP-2 is remaining bound to chitosan and calcium within the microspheres. During the osteogenic studies, it was also determined that sampling involving multiple day intervals underestimating the amount of BMP-2 released from the scaffold. This is attributed to degradation of the BMP-2 molecule in solution. Therefore, collecting elutates everyday may be a more accurate measure of BMP-2 elution. Future studies could tailor this elution to maximize the benefit of BMP-2 with delayed and prolonged release at a therapeutic level.

Another finding of this study was that BMP-2-responsive cells did not respond equally when in the presence of varying concentrations of vancomycin. Cells exposed to the highest level of BMP-2 in the presence of 3600-112ug/ml vancomycin exhibited ~20-50% less ALP activity than control cells exposed only to BMP-2. This indicates that vancomycin has an inhibitory effect on BMP-2 and thus could affect the wound healing process. If the scaffold were to elute the highest levels of vancomycin first, and delayed release BMP-2 were achieved during this time then inhibition of the BMP-2 would theoretically be minimized.

Further work for this study will:

- Investigate the bioactivity of elutate samples from the microspheres on a human osteosarcoma cell line.
- Determine the amount of BMP-2 remaining in the microsphere.
- Assess changes in viability of the cells exposed to mock elutates and determine if they are due to toxicity of the therapeutic agents used or to cell induced apoptosis.

<u>So What</u>

This scaffold represents a point-of-care device, which can be tailored by the surgeon to contain antibiotics or growth factors. Antibiotic loading resulted in prolonged bacterial killing which is an ideal characteristic for a traumatic musculoskeletal injury. Extended release of BMP-2 was acquired, but may need to be increased to ensure a clinically effective dose. Devices such as this will provide patients with the best care because the device can be tailored directly to their needs, eliminating unnecessary therapeutics and potential toxicity issues, while reducing infection, decreasing recovery times, revision surgeries and hospital stays.

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APPENDICES– See below.

SUPPORTING DATA: (included in report text)

Appendix I

Effectiveness of a Dual Drug Delivery Calcium Sulfate, Chitosan-Calcium Phosphate Bone Scaffold.

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Statement of Purpose

Infections resulting from wound contaminations can lead to delayed healing, high medical expenses, increased site morbidity and patient mortality. Studies have found as much as 65-70% of open musculoskeletal wounds can be contaminated with microorganisms.[1,2] Therefore, there is a need for orthopedic scaffolds to be drug delivery vehicles in addition to having good wound healing properties. This study investigated known scaffold materials: chitosan, calcium phosphate (CaP) and calcium sulfate (CaS) as a drug delivery system for dual antibiotic release. The goals of this study was to extend the antibiotic release of two commonly used orthopedic drugs, Vancoymcin and Amikacin, from the scaffold material for up to six weeks at a level that would abate and kill bacterial species.

Methods

Composite Bead Fabrication: A co-precipitation method was employed for the fabrication of the composite chitosan-CaP beads.[3] Solution of 3.57 wt% chitosan (80% DDA, Prim-Ex), 0.1 M $CaCl_2$, 0.06 M NaH_2PO_4, (Ca:P ratio = 1.67) in 2 wt% acetic acid was dripped into a solution of 20% NaOH, 30% methanol, 50% water (pH=13) to precipitate beads. After 24 hours (hrs) the beads were neutralized, frozen (-20 ^oC) and lyophilized in a 2.5 liter Labconco freeze-dryer for 48 hrs.

Amikacin and Vancomycin Loading: Approximately 80 mg of composite beads were loaded in 1.5 mL of a 10 mg/mL solution of amikacin at room temperature (RT) for 24 hrs. The beads were removed and placed in either clean vials with 2 mL of PBS at 37^oC or mixed with CaS to form pellets. A vancomycin solution was added to CaS powder to yield 2% (w/w) of vancomycin in the pellet. Three scaffolds were analyzed (n=5): A = amikacin loaded beads, V = vancomycin loaded CaS and VA = amikacin beads coated by vancomycin CaS.

Pellet Fabrication and Elution: The pellets were made from 0.6g of α-hemihydrate CaS and 0.24mL dI H_2O. Composite beads were mixed into some CaS samples prior to setting. Dried scaffolds in 1.5mL PBS were sampled at 1, 5, 12, 24, 48, 96, 84, 168, 336, 480, 648, 816 and 1008 hrs. The PBS solution was completely refreshed after each elution time point. The samples were analyzed via a fluorescent immunoassay with the TDx machine (Abbott Diagnostics, Abbott Park, IL).

Inhibition of Growth and Bactericidal Assay: Sterile tubes were prepared with 1.75 ml of Trypticase soy broth (TSB), 200 µl eluate sample or 200 µl PBS and 25-50 µl of *S.aureus* Cowan I and *P. aeruginosa* ATCC 27317 grown to ~2x10^6 CFU or 25-50 µl PBS for blanks. Samples were grown ON at 37 °C and the absorbance at 530 nm was recorded. All samples which contained no growth were diluted 10, 100 and 1000 times, and 100 µl was plated on TSB plates and incubated ON at 37 °C.

Results

The scaffold (VA) loaded with both drugs showed the longest elution profile with release above vancomycin MIC for 6 wks and amikacin MIC for 27days compared with the other scaffolds loaded with only one antibiotic.

Table 1: Vancomycin and Amikacin Elution from scaffold material for 6 weeks

	Amikacin (µg/mL)		Vancomycin (µg/mL)	
Hrs	VA	A	VA	V
1	227 ± 21	534 ± 48	2970 ± 150	4250 ± 1790
5	176 ± 22	193 ± 9	1900 ± 186	2410 ± 206
12	158 ± 16	56.2 ± 1.9	1370 ± 144	2050 ± 93
24	111 ± 7	22.0 ± 0.3	1260 ± 70.7	1880 ± 104
48	68.5 ± 12.7	11.6 ± 0.3	1340 ± 116	1730 ± 109
96	29.2 ± 4.4	5.44 ± 0.16	1580 ± 108	626 ± 84
168	14.9 ± 2.2	3.00 ± 0.06	1200 ± 114	113 ± 13
336	9.71 ± 0.72	2.04 ± 0.06	525 ± 114	26.7 ± 3.7
480	6.26 ± 0.48	2.00 ± 0.10	148 ± 34	7.20 ± 0.77
648	4.13 ± 0.32	1.69 ± 0.11	46.1 ± 9.51	4.53 ± 0.39
816	3.19 ± 0.32	2.00 ± 0.30	16.1 ± 3.29	3.22 ± 0.39
1008	3.31 ± 0.30	1.84 ± 0.12	8.33 ± 1.42	3.70 ± 0.11

Vancomycin in combination with amikacin (VA) inhibited bacterial growth up to 34 days and was bactericidal for 27days. Vancomycin (V) alone inhibited and killed bacteria for 14 days. Amikacin (A) was less effective against *P. aeruginosa* for both samples with inhibition through 24hrs and bactericidal only for the first hour.

Table 2: Inhibitory/bactericidal effect of vancomycin and amikacin against *S. aureus* or *P.aeruginosa* respectively. (-) indicates no growth.

	S. aureus				*P. aeruginosa*			
	Inhib.		Bact.		Inhib.		Bact.	
Hrs	V	VA	V	VA	A	VA	A	VA
1	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	+
12	-	-	-	-	-	-	+	+
24	-	-	-	-	-	-	+	+
48	-	-	-	-	+	+	+	+
96	-	-	-	-	+	+	+	+
168	-	-	-	-	+	+	+	+
336	-	-	-	-	+	+	+	+
480	+	-	+	-	+	+	+	+
648	+	-	+	-	+	+	+	+
816	+	-	+	+	+	+	+	+
1008	+	+	+	+	+	+	+	+

Conclusions:

The scaffold containing both antibiotics exhibited extended drug release and bacterial abatement and killing of *S.aureus*. The scaffold was not as effective against *P. aeruginosa,* most likely due to the smaller amount of amikacin loaded into the scaffold. It appears that there may be an interaction between vancomycin, amikacin and the scaffold that extends the elution profile over the single drug delivery scaffolds. Future studies will increase the amount of amikacin in the scaffold to increase activity against *P.aeruginosa* as well as loading other factors into the scaffold which may be helpful for bacterial abatement or bone growth.

Acknowledgements: Telemedicine and Advanced Technology Research Center at the U.S. Army Medical Research and Materiel Command through award W18XWH-09-1-05796

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