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Inductive Microenvironment for Improved Osseous Integration

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14. ABSTRACT The ultimate goal of tissue engineering is to address the current organ shortage problem, i.e. development of an alternative therapeutic strategy to autografting and allografting. Several approaches are currently been used to regenerate damaged tissue using the principles of tissue engineering. Among these, scaffold based tissue engineering wherein, biomaterials are used alone or in combination with cells and biologically active molecules is one of the most attractive and extensively investigated approaches. One of the most important challenges is to ensure proper in vivo performance of the engineered cell-scaffold constructs under challenging microenvironmental conditions of inflammation characteristic of damaged tissues. This calls for the development of cell instructive regenerative biomaterial structures that can communicate with host cells via multiple regulatory signals. The present research project aims to investigate the ability of an injectable matrix from a biodegradable polymer "Chitosan" in modulating the host tissue microenvironment to promote regeneration. The overall objective of this study is to develop strategies to improve regeneration of critical size bone defects by creating an optimized inductive microenvironment using an injectable chitosan carrier with cells and rhBMP-2 alone or in combination. The report discusses the studies we performed over the past one year towards evaluating injectable compositions from chitosans of different degree of acetylations in vitro using mouse macrophages and in vivo using mouse calvarial and segmental bone defect model.					
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INTRODUCTION:

The ultimate goal of tissue engineering is to address the current organ shortage problem, i.e., to develop an alternative therapeutic strategy to autografting and allografting. Among the several methods currently under development, scaffold based tissue engineering, wherein, biomaterials are used alone or in combination with cells and factors is one of the most attractive approaches. Several biological and engineering challenges still remain for the successful clinical translation of the laboratory research to make tissue engineering a reliable route for organ/tissue regeneration. One of the most important challenges lies in our effort to ensure proper in vivo performance of the engineered cell-scaffold constructs under the challenging microenvironmental conditions of the damaged tissue. Cell instructive regenerative biomaterials that could interact with host/donor cells to promote regeneration could significantly improve the clinical outcome. The aim of the proposed research is to investigate the ability of an injectable matrix from a biodegradable polymer “chitosan” of different chemistries in modulating the host-tissue microenvironment to promote regeneration. The hypothesis behind the study was that the localized inductive microenvironment presented by the injectable chitosan matrix along with mesenchymal stem cells and /bone morphogenetic protein-2 will significantly enhance the engraftment of donor cells and promote host mediated regenerative events thereby leading to improved quality and quantity of the regenerated bone.

During the first year of the study, we focused on developing and characterizing chitosan's of different degree of deacetylation, studied the effect of chitosan degree of deacetylation on macrophage genotype, and optimized the implantation method to implant injectable chitosan gel in mouse calvarial defect model.

During the past one year, we extended the in vitro studies to understand the phenotype of the macrophages exposed to chitosans of different degree of deacetylations. One of the issue we have faced was in developing the Cx3cr1-EGFP/cd-11c-mCherry reporter mouse to follow the immune response in vivo. We are still in the process of optimizing the protocol, however, in the past year we also developed immunohistochemical methods to understand the cellular activities towards chitosans of different degree of deacetylation. Another issue we faced last year was the lose of the post doctoral fellow who was involved in this project. We hired a new post doctoral

fellow and she is currently trained and up to speed to successfully complete the proposed project. We also finished the initial calvarial studies demonstrating the feasibility of localizing the injectable scaffold in the calvarial defect, followed cellular response as a function of time to chitosans of different degree of deacetylation to demonstrate its ability to support host cell infiltration and biocompatibility. We did preliminary studies to demonstrate the feasibility of using injectable chitosan to locally deliver rhBMP-2. We also optimized the critical size long bone defect model in mice for injectable gel to study the quality of the regenerated bone in the presence of implanted cells and rhBMP-2. We will be starting the implantation studies in November of 2012 to follow the extent of healing and the biomechanical properties of the resulting calvarial and long bone defect model in the presence of transplanted cells and rhBMP-2

BODY:

Specific aim 1; Task I: Preparation of chitosans with different degree of deacetylations via the acetic anhydride method

Status: Completed and presented in previous progress report dated 10/26/2011

Specific aim 1; Task II: Acquire IACUC followed by USAMRMC ACURO approval for the proposed animal studies

Status: Completed and presented in previous progress report dated 10/26/2011

Specific aim 1; Task III: Characterization of the chitosans via chromatography and spectrophotometry

Status: Completed and presented in previous progress report dated 10/26/2011

Specific aim 1; Task IV: Development of injectable composition from chitosans having different degree of deacetylation

Status: Completed and presented in previous progress report dated 10/26/2011

Specific aim 1; Task V & VI: Generation of transgenic mice with SMAA-mCherry/Tie2-Citrine and Cx3cr1-EGFP/cd-11c-mCherry reporters.

We are still in the process of developing the model with all the required markers. However, to move forward with the study to understand the different cell types involved in chitosans of different degree of deacetylation, we are currently optimizing the immunohistochemical techniques (page 19-21).

Effect of chitosans of varying degree of deacetylation on mouse macrophage cells:

Methods

In vitro studies were performed to evaluate whether chitosans with different degree of deacetylations will behave differently towards macrophage cells. The study evaluated the effect of chitosan degree of deacetylation on macrophage morphology and cytokine release from macrophage cells to understand the immunomodulatory effect of chitosans of different chemistry.

Briefly, chitosans of varying degree of deacetylation were dissolved in 1% acetic acid at a concentration of 1% w/v and then autoclaved. Chitosan films were prepared by adding 0.3ml of the chitosan solution per well in a 24 well plate and drying overnight in biosafety cabinet. After complete drying of chitosan films, 1ml of 0.1N sodium hydroxide was added in to each well for 1h for neutralizing the acetic acid traces, if any. The wells were then washed two times with sterile PBS and then two times with RPMI medium, to ensure the complete removal of traces of acid.

For morphological evaluation of macrophages on chitosan films, RAW264.7 cells were seeded on chitosan films at a seeding density of 40,000 cells per well and cultured for 24h (n=4). After 24h, photomicrographs were taken at various magnifications using Olympus CKX41 equipped with Qcolor5 digital camera. Further, cell proliferation on chitosan films was studied using CellTiter96® non-radioactive cell proliferation kit (Promega). Briefly, 100µl of dye solution and 400µl of complete medium was added into each well after 24h of culture. The plate was then incubated at 37°C for 2h after which 500µl of stopmix was added to each well. The formazan crystals were allowed to dissolve for around 30min, mixed thoroughly. About 500µl of the

supernatant was transferred to a 48well plate and the absorbance was taken at 570nm, as a measure of cell survival.

For determining the immunomodulatory effect of chitosans of varying degree of deacetylations, cytokine release profile of mouse macrophages was studied. The cytokines were determined based on our previous study evaluating macrophage genotype when exposed to chitosans of different chemistries. In his study, RAW264.7 cells were seeded on chitosan films at a seeding density of 500,000 cells per well (0.5ml media) and cultured for 24h. After 24h, the medium was collected, centrifuged at 12000g for 4 min and supernatant was transferred into prelabelled microcentrifuge tubes and stored at -70°C, till further analysis. Quantitative ELISA kits (R&D Systems) were used to determine the expressions of Mouse IL-10 and Mouse TNF- α in the supernatants, as per manufacturer's protocol.

Results:

The morphology of mouse macrophages (RAW264.7 cell line) on chitosans of different degree of deacetylations studied by optical microscopy and compared to tissue culture treated surface as control. On TC-control, the macrophages were found to adhere very well as well as spread to show spindle morphology. The chitosan films irrespective of the chemistry significantly decreased macrophage spreading. In case of chitosans obtained from Carbomer, Inc. (C- series), it was observed that at lower degree of deacetylations, cells remained in large aggregates and maintained a round morphology. With increasing degree of deacetylation, however, the aggregation reduced (C-28.88% - C-71.99% films.) On further increasing the degree of deacetylation, the cellular aggregation reduced, however the cells still retained a round morphology (Figure 1).

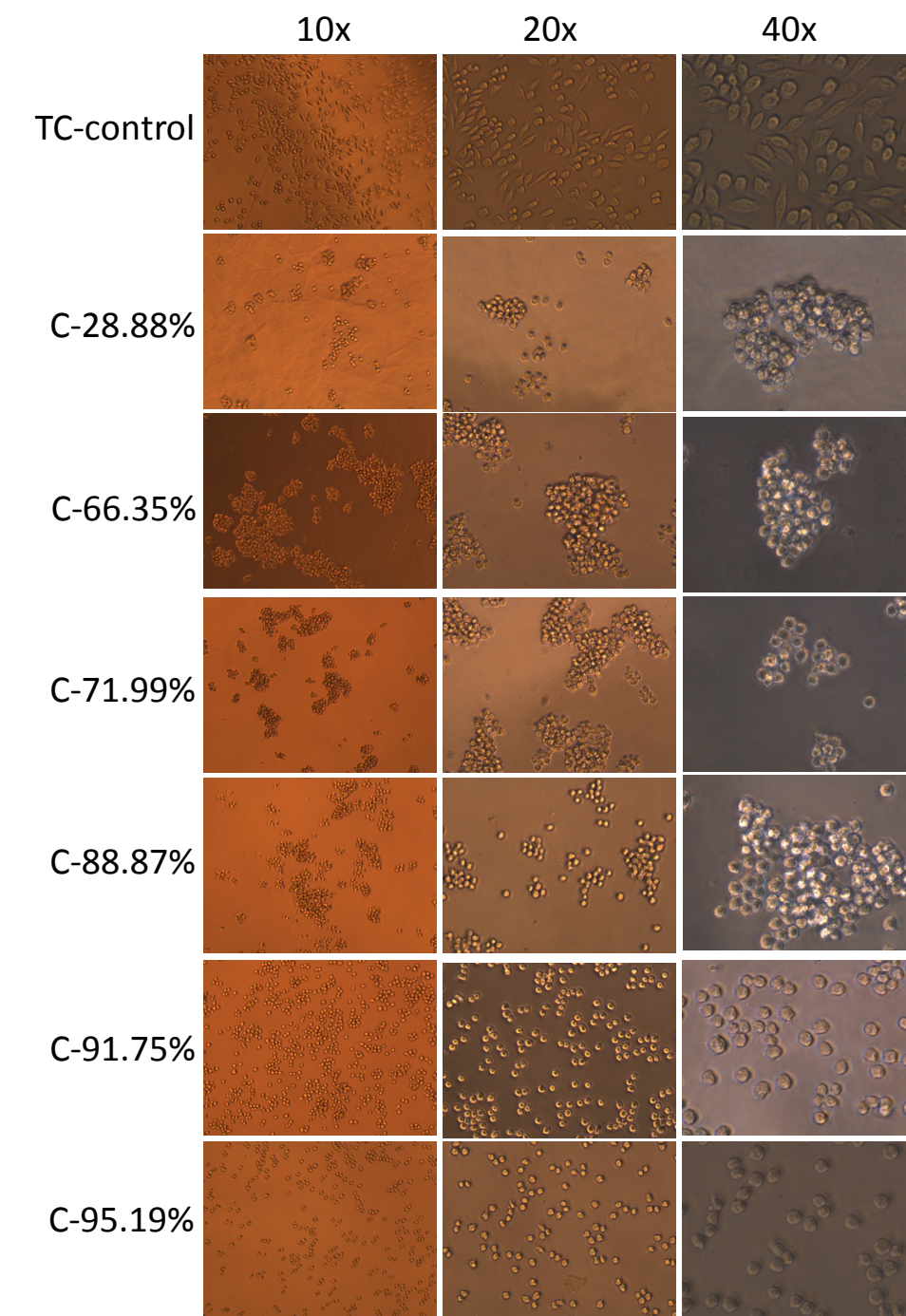


Figure 1. Light microscopy showing morphology of macrophage cells (RAW 264.7) cells exposed to chitosan (CarboMer, Inc.) films of different degree of deacetylations for 24h

Similar trend was found with chitosan procured from Biosyntech, Inc. Irrespective of the degree of deacetylation, the cells on chitosan film retained the rounded morphology and cell aggregation decreased at higher degree of deacetylation (Figure 2).

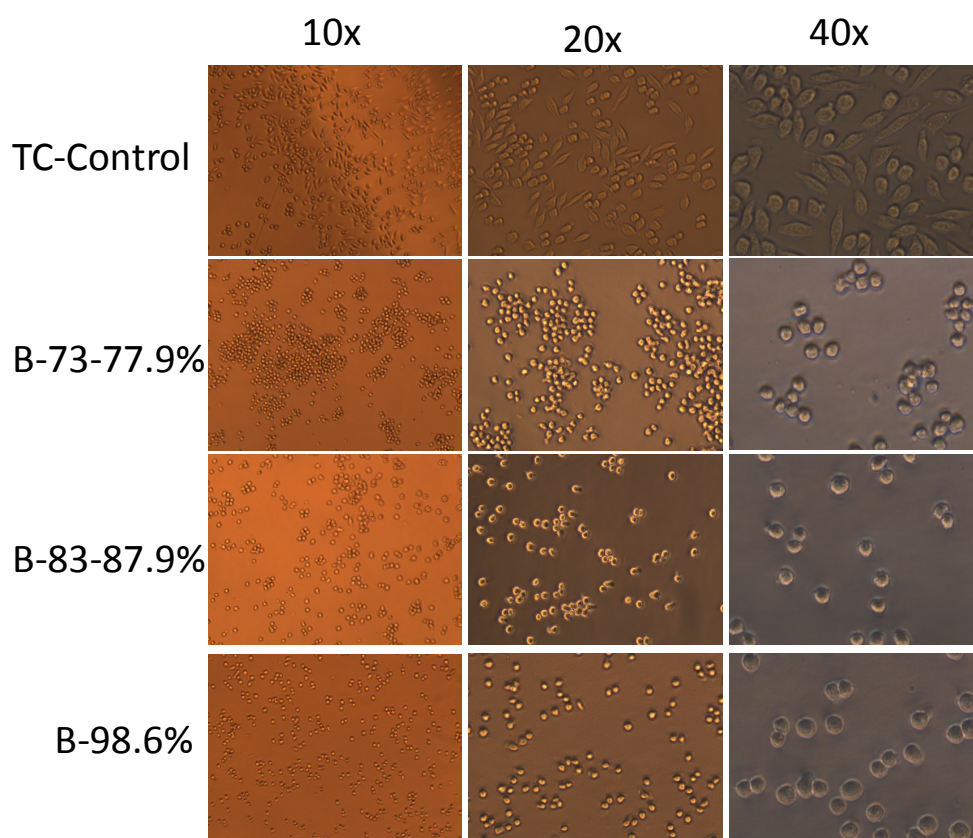


Figure 2. Light microscopy showing morphology of macrophage cells (RAW 264.7) cells exposed to chitosan (Biosyntech Canada, Inc.) films of different degree of deacetylations for 24h

The difference in cell morphology and aggregation in presence of chitosan films may have an impact on macrophage survival, and cytokine release profile. The viability of the cells was followed by the standard MTT assay. Chitosans with lower degree of deacetylation from carbomer showed decreased cell viability compared to higher degree of deacetylation. Only

chitosans with higher degree of deacetylation 73-98% was evaluated for biosynthetic chitosans and all of them showed significant cellular viability similar to the control (Figure 3).

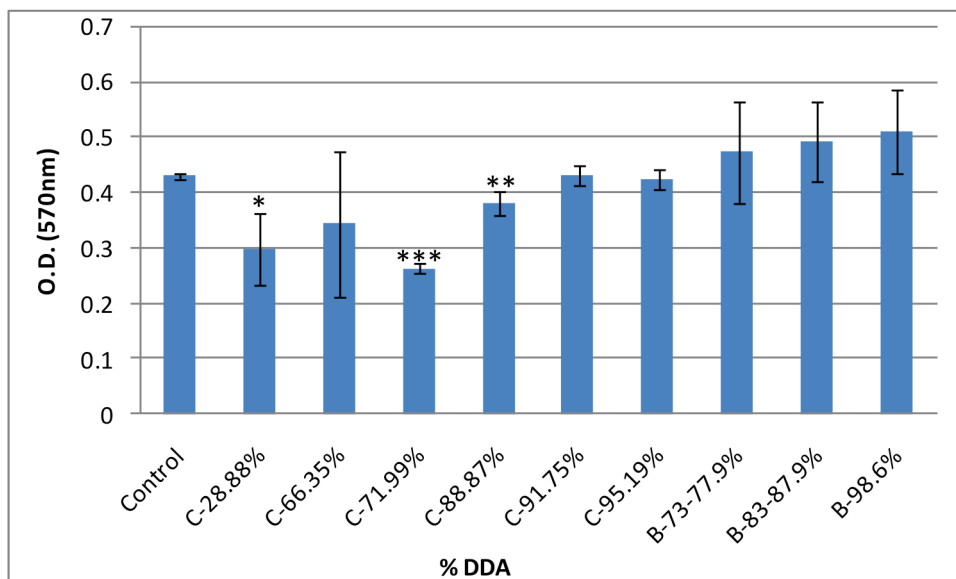


Figure 3. MTT absorption of macrophage cells (RAW 264.7) cells exposed to chitosan films of different degree of deacetylations for 24h

***P<0.001, **P=0.018, *P=0.025 Vs Control (t-test); n=3

To further understand the effect of chitosan chemistry on cytokine release, the expression of various cytokines was studied by ELISA. Our previous study (last report) showed significant increase in TNF- α messenger RNA expression by macrophages exposed to chitosans of higher degree of acetylation. Here we evaluated the protein expression using ELISA and it followed similar trend using carbomer chitosan. The chitosan with 95.19% degree of deacetylation showed the highest TNF- α expression. The biosynthetic chitosan with degree of deacetylation upto 87.9% showed very low TNF- α protein expression. However the chitosan with 98.6% degree of deacetylation showed significant increase in TNF- α expression compared to the control (Figure 4). This is consistent with a recent study correlating macrophage morphology to the extent of TNF- α production [1]. The study demonstrated less TNF- α with rounded cells.

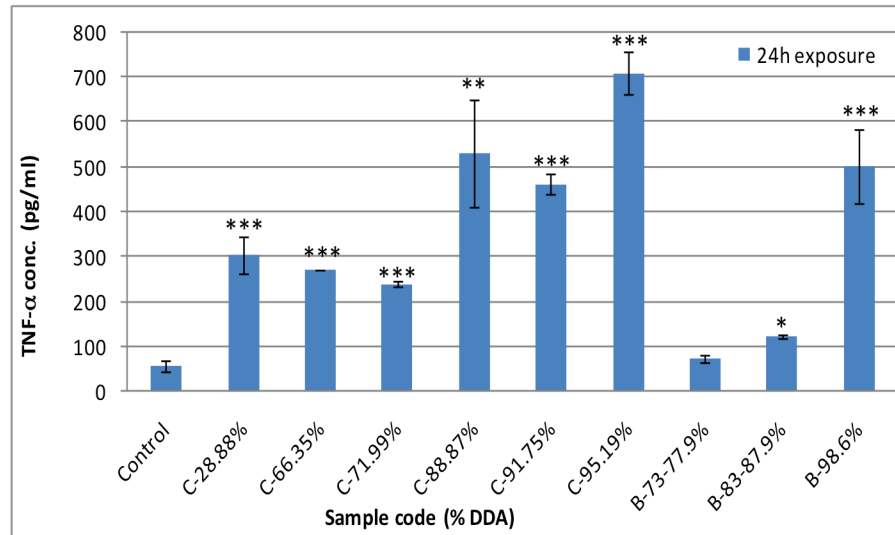


Figure 4. TNF- α cytokine expression of macrophage cells (RAW 264.7) cells exposed to chitosan films of different degree of deacetylations for 24h
 ***P<0.001; **P=0.002, *P=0.001 Vs Control (t-test); n=3

The other interleukins currently been investigated included IL-6, IL-10 and IL-4. Since the biosynthetic chitosan with degree of deacetylation in the range of 73-87% and 98.6% showed significant differences in cell behavior, those two degrees of deacetylation has been selected for further studies to systematically evaluate the in vivo response of these two groups of chitosans using calvarial and long bone defect models.

Specific aim 2; Task I: Generation of dual fluorescent protein reporter mice and isolation of bone marrow stem cells

Col3.6cyan mice are being bred for isolation of blue fluorescent bone marrow derived mesenchymal stem cells which shall be used for implantation in Col3.6tpz (green) mice using calvarial and long bone defect models to understand the role of host and donor cell contributions in supporting bone formation using chitosans of different chemistries by following the fluorescent reporters.

Specific aim 2; Task II: Implantation of various compositions in critical size cranial defects using reporter mice and following the cellular response via histology, histomorphometry, and

immunohistochemistry and quality and quantity of regenerated bone via microCT and biomechanical testing

Methods

Surgical procedure for cranial defect

The mouse was anesthetized and then placed in a prone position and the cranium shaved for fur, and washed with betadine and swabbed with alcohol. A ~1cm incision was made on the skin on top of the head. The periosteum was then incised over the parietal bone in the frontal plane with a scalpel blade and reflected with a periosteal elevator. Central 3.5 mm diameter holes were created through each of the two parietal bones using a trephine, without involving any of the cranial sutures. Healos was used as a control in these studies to evaluate the efficacy of implanting the pregelled chitosan compositions compared to a preformed scaffold.

Here, chitosan gels (Biosynthech, Inc.) with two different degree of deacetylations viz. 73-77% and 98.6 (labeled as B-73-77% and B-98.6%, respectively) were tested. Chitosan-AHP solutions were prepared as described before. 10-15 μ l cold chitosan-AHP solutions were used to fill in the cranial defects. Time points used were 4 and 8 weeks. Xylenol orange solution was injected one day before sampling to label newly formed bone in red. Samples were frozen in Cryomatrix solution (Thermo Scientific, USA) for cryo-histology.

Our pilot study (previous report) presented challenges in terms of retaining the injected gels at the site of implantation while closing the skin. Therefore, the protocol was modified and performed or pregelled chitosan compositions were used for these studies. Briefly, 10-15 μ l chitosan-AHP solutions were drawn into the syringe and then were incubated at 42-45°C for 15-20 minutes before injected into the defect site.

Additionally, effect of BMP-2 addition on bone formation was studied by adding BMP-2 in both B-98.6% and healos before implantation into the calvarial defects.

Results

Two chitosan samples with different degrees of deacetylations viz. B-73-77% and B-98.6% were tested *in vivo* to evaluate their ability to support host cell infiltration using calvarial bone defect model. In our previous studies (last year report), we encountered challenges with regards to maintaining the gel at the site of implantation. The major reason for the poor gelation *in vivo*

has been identified as the lower body temperature at the implantation site, since the thermogels were designed to gel at 37°C. Hence, the method of implantation was modified, wherein the gelling solution was kept at 37°C before implantation to increase the viscosity of the injected chitosan and the process successfully helped in retaining the gels at the site of implantation.

Figure 5 shows the histological sections of parietal bone implanted with chitosan with degree of deacetylation of 73-77% degree of deacetylation 4 weeks after implantation. Commercially available Healos was used as the positive control. The photograph (panel F) shows the feasibility of retaining the chitosan gel at the defect site at 4 weeks post implantation. The Panel A shows the fluorescent images of the sections showing the mineralized host bone. The chitosan thermogel or healos by itself did not promote any significant increase in the bone formation. Panel B shows the same section without the mineralized bone content to clearly evaluate the presence of fluorescent host osteoblast cells surrounding the defect area in both healos and chitosan thermogels. The micrograph showed only very low levels of intense green fluorescence which is characteristic of osteoblast cells. In addition to this, the presence of light green cells which is characteristic of fibroblast cells were also not evident within the chitosan scaffold at this time point. Panel C shows the trap staining of the slides to evaluate the osteoclast activity. Not much osteoclast activity was observed in both the samples. Panel D shows the alkaline phosphatase activity. The chitosan scaffold did show increased alkaline phosphatase activity along the edges. Panel E shows the H&E stained sections showing the overall cellular activity within and around the cells. As shown in the figure both Healos and chitosan gel showed significant cellular infiltration as indicated by the blue nuclear stain. This indicates the infiltration of immune cells such as macrophage cells within the scaffolds, since the extent of osteoblastic and fibroblastic infiltration was found to be very low as evident from Panel B.

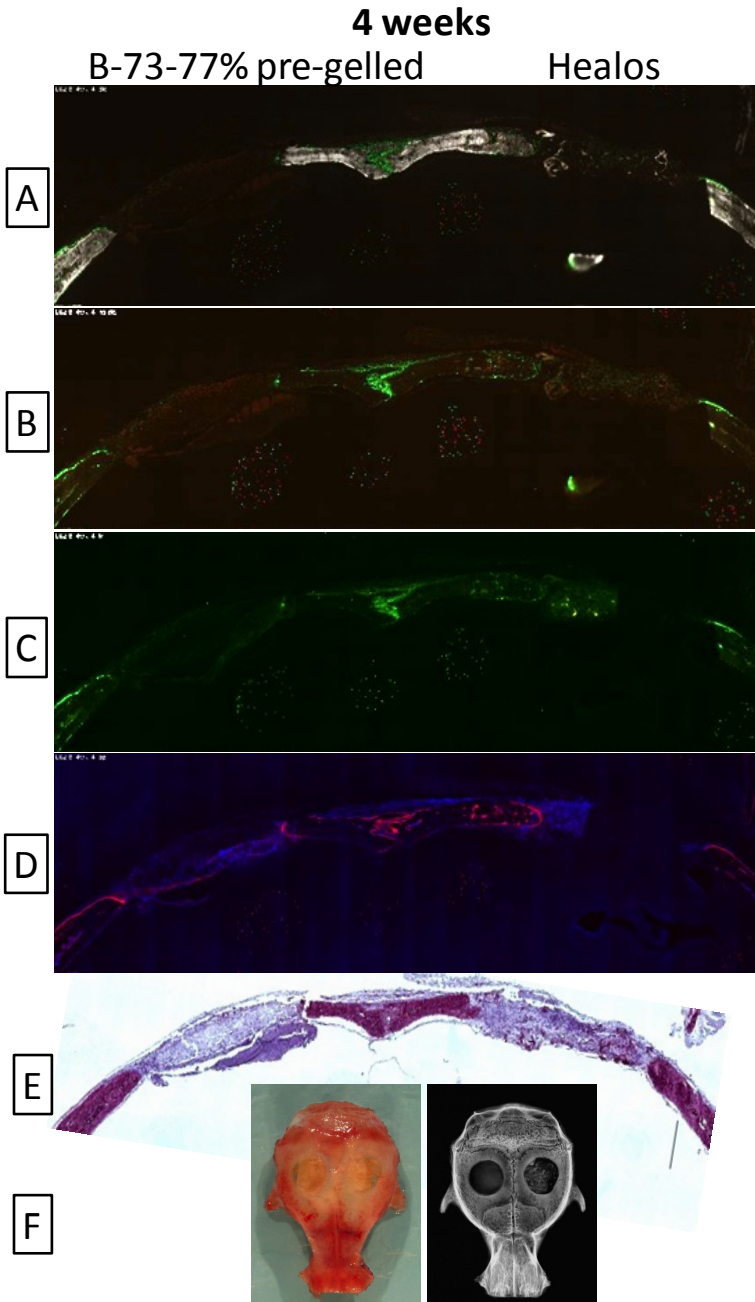


Figure 5. A) Fluorescent images showing mineralized bone; (B) shows image A without the mineral content C) TRAP staining indicating the osteoclast activity D) Alkaline phosphatase staining indicating osteoblast activity in the region; (E) H&E stained histological section F) x-ray & photographs of mouse calvaria post implantation

Figure 6 shows the 73-77% chitosan implants after 8 weeks post implantation. Panel A shows the micrograph of the mineralized bone and Panel B shows the micrograph without the

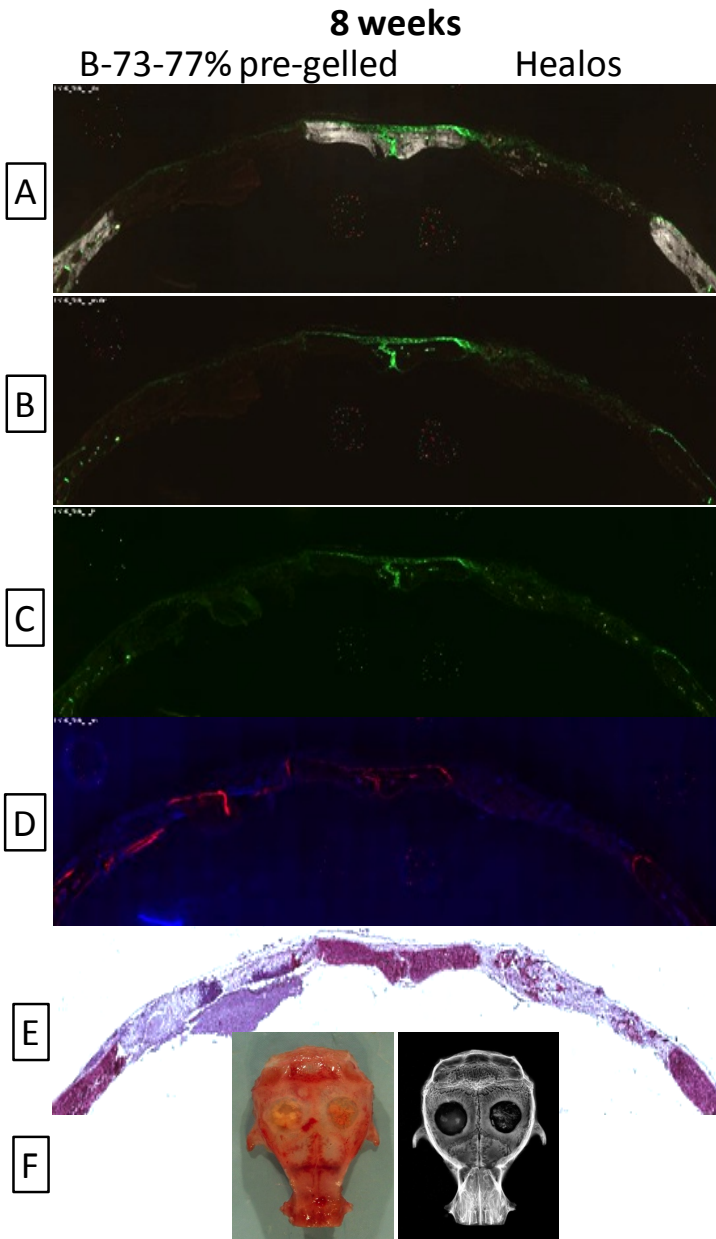


Figure 6. A) Fluorescent images showing mineralized bone; (B) shows image A without the mineral content C) TRAP staining indicating the osteoclast activity D) Alkaline phosphatase staining indicated osteoblast activity in the region; (E) H&E stained histological section F) x-ray & photographs of mouse calvaria post implantation

mineral content. As in the case of 4 week time point, not much infiltration of osteoblastic or fibroblastic lineage cells were observed at this time point as well. Panel C shows the trap stain also did not show significant increase in osteoclastic activity in chitosan implant. Panel D shows

the alkphos activity and as in the case of 4 weeks, some increase in Alk phos staining in chitosan scaffold. Panel E shows the H&E indicating the presence of polymer even though some polymer degradation is evident compared to 4 weeks. Similar was the case with Healos. Both sides showed the presence of cellular infiltration most probably macrophage like cells since the presence of much osteoblast and fibroblast was not highly evident from Panel B.

Figure 7 shows the histological sections of parietal bone implanted with chitosan of degree of deacetylation of 98.6% degree of deacetylation 4 weeks after implantation. Healos was used as the positive control. As in the case of 73-77% chitosan, the pre-gelled injectable gel developed from 98.6% was also were able to be retained at the sight of implantation as shown in photograph (panel F). The Panel A shows the fluorescent images of the slides with mineralized host bone. Compared to Figure 5, the chitosan gel in Figure 7 showed improved cellular infiltration as evidenced from light green fluorescence within the gel (Panel B). Panel C shows the trap staining of the slides to evaluate the osteoclast activity. Not much osteoclast activity was observed in the chitosan implant. Panel D shows the alkaline phosphatase activity As in the case of Figure 5 some alkaline phosphatase activity was observed in the chitosan implant. Panel E shows the H&E stained sections showing the overall cellular activity within and around the cells. As shown in the figure both Healos and chitosan gel showed significant cellular infiltration as indicated by the blue nuclear stain. This indicates the infiltration of macrophage cells within the scaffolds, along with fibroblastic infiltration as evidenced from less intense green fluorescence within the chitosan implant as indicated in Panel B. In summary, the pre-gelled thermogels developed from 98.6% chitosan gels was retained at the defect site and the chitosan gel did support some host cell infiltration.

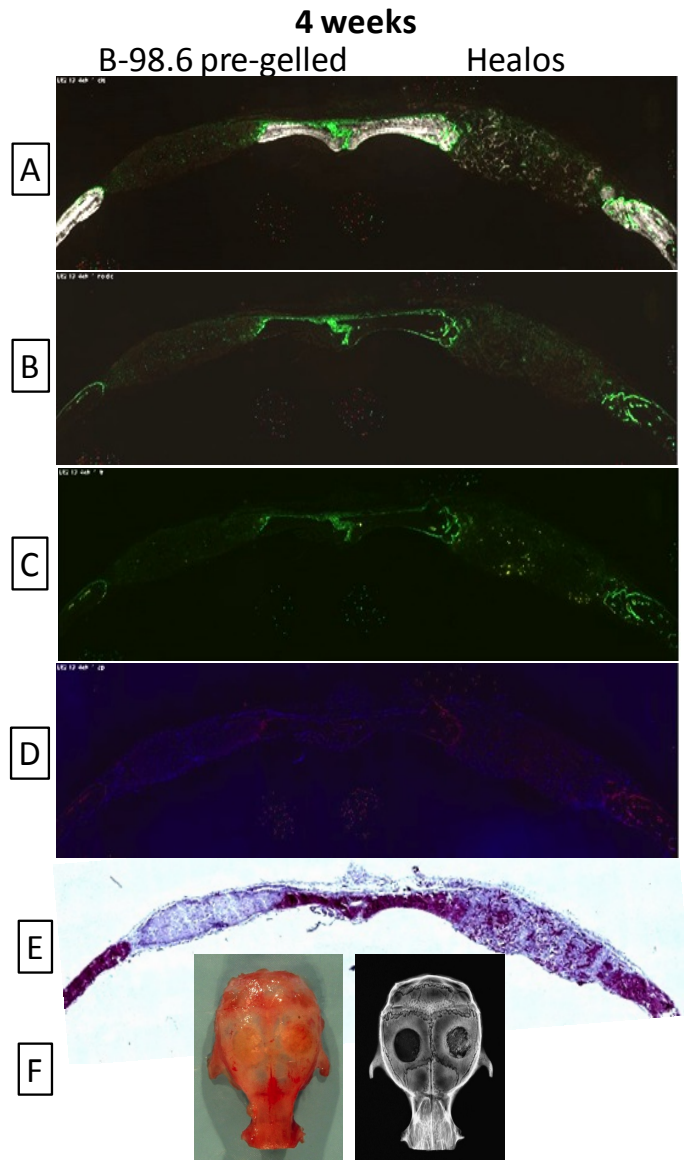


Figure 7. A) Fluorescent images showing mineralized bone; (B) shows image A without the mineral content C) TRAP staining indicating the osteoclast activity D) Alkaline phosphatase staining indicated osteoblast activity in the region; (E) H&E stained histological section F) x-ray & photographs of mouse calvaria post implantation

Figure 8 shows the 98.6% chitosan implants after 8 weeks post implantation. Panel A shows the micrograph of the mineralized bone and Panel B shows the micrograph without the

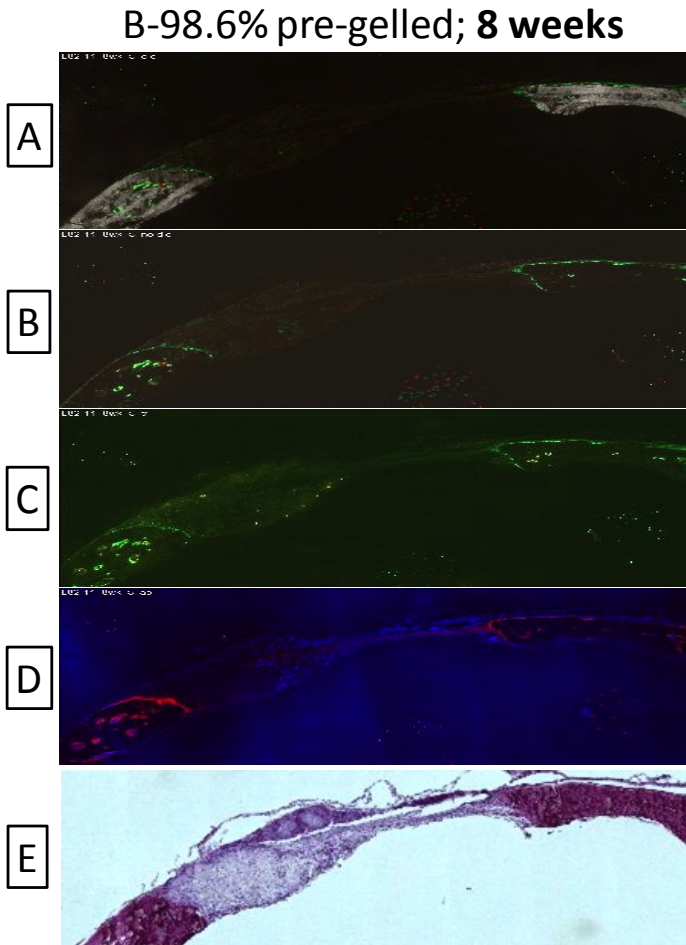


Figure 8. A) Fluorescent images showing mineralized bone; (B) shows image A without the mineral content C) TRAP staining indicating the osteoclast activity D) Alkaline phosphatase staining indicated osteoblast activity in the region; (E) H&E stained histological section

mineral content. As in the case of 4 week time point, some host cell infiltration was evident. Panel C shows the trap stain and some osteoclast activity was also evident. However, even after 8 weeks, the chitosan was not completely degraded. Panel D shows the alkphos activity and the stain is evident on the ends the defect. Panel E shows the H&E indicating the presence of polymer at the implantation site.

Immunohistochemical analysis of the immune response towards injectable chitosan gel developed from 98.6% deacetylated chitosan: (Specific aim 1; Task V & VI)

Methods

Calvarial defects were generated as described in Specific aim 2; Task II. Immuno-histochemistry was performed to understand the effect of chitosan degree of deacetylation on host immune response, at both 4 weeks and 8 weeks time points. The following protocol was used:

1. The cryosectioned calvarial defect slides were rinsed two times with PBS for 5 min each.
2. The sections were then incubated with 0.1% Triton – PBS for 30min, then rinsed with PBS and further incubated with 1xPower Block at RT for 20min.
3. The sections were then rinsed three times with PBS and placed in Rat anti-Mouse F4/80 (BioLegend Cat# 123101) diluted to 1:100 with 0.5% BSA in PBS at RT for 1 hour or 4°C overnight.
4. The sections were then rinsed in PBS three times and then incubated with Alexo Fluor 594 Goat anti-Rat IgG (Vitrogene Cat # A11007) 1:500 in PBS with 1% NGS and 0.5% BSA, incubate at RT for 1 hour.
5. The sections were finally rinsed with PBS three times and then covered with 50% glycerin for viewing under fluorescent microscope.

Results:

Figures 9 and 10 show the immunohistochemical evaluation of chitosan (B-98.6%) for determination of immune response of implanted chitosan in mouse calvarial bone defect model. In col3.5tpz mice, fibroblasts show a mild green color where as osteoblasts show a bright green fluorescence. F4/80 is a marker of mature macrophages and the hence the macrophages are stained red. The nuclei were stained blue with DAPI. As shown in Figure 9, at 4 weeks, significant cell infiltration is evident from DAPI stains which are mostly macrophages as evident from Figure 9C. Some host fibroblast and osteoblast were also evident at 4 weeks. A 8 weeks, Figure 10, the number of green cells significantly increased showing increased host fibroblast and osteoblast cell infiltration into the chitosan gel along with macrophages. We are currently in the process of identifying the phenotype of the macrophages to study if the environment can modulate and inflammatory or antiinflammatory phenotype of the macrophages.

B-98.6% pre-gelled_4 weeks

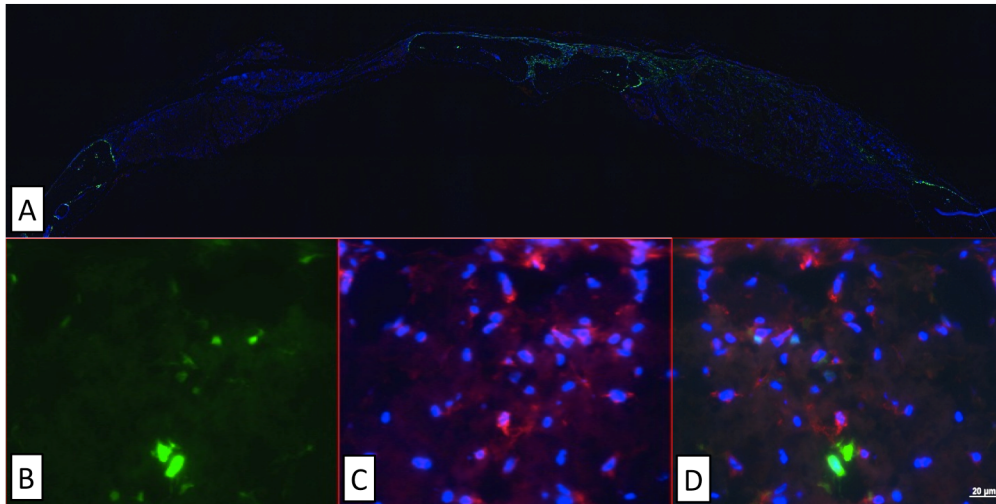


Figure 9 F4/80 expression in B-98.6% implanted calvarial defect cryosections A) DAPI stained section (A) complete cryosection B) green autofluorescence of host (Col3.6tpz) cells, 20x C) DAPI stained section, 20x D) overlay of B & C, 20x

B-98.6% pre-gelled_8 weeks

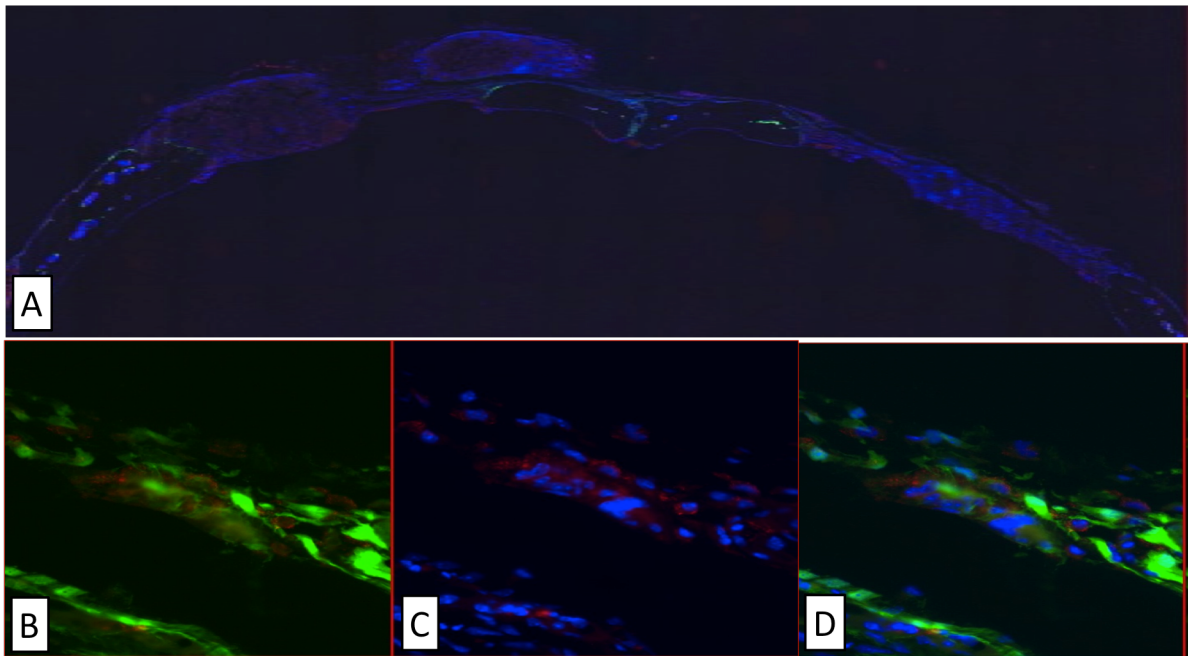


Figure 10. Immunohistochemistry staining studies showing F4/80, a marker for mature mouse macrophages (red) (A) complete cryosection B) green autofluorescence of host (Col3.6tpz) cells, 20x C) DAPI stained section, 20x D) overlay of B & C, 20x

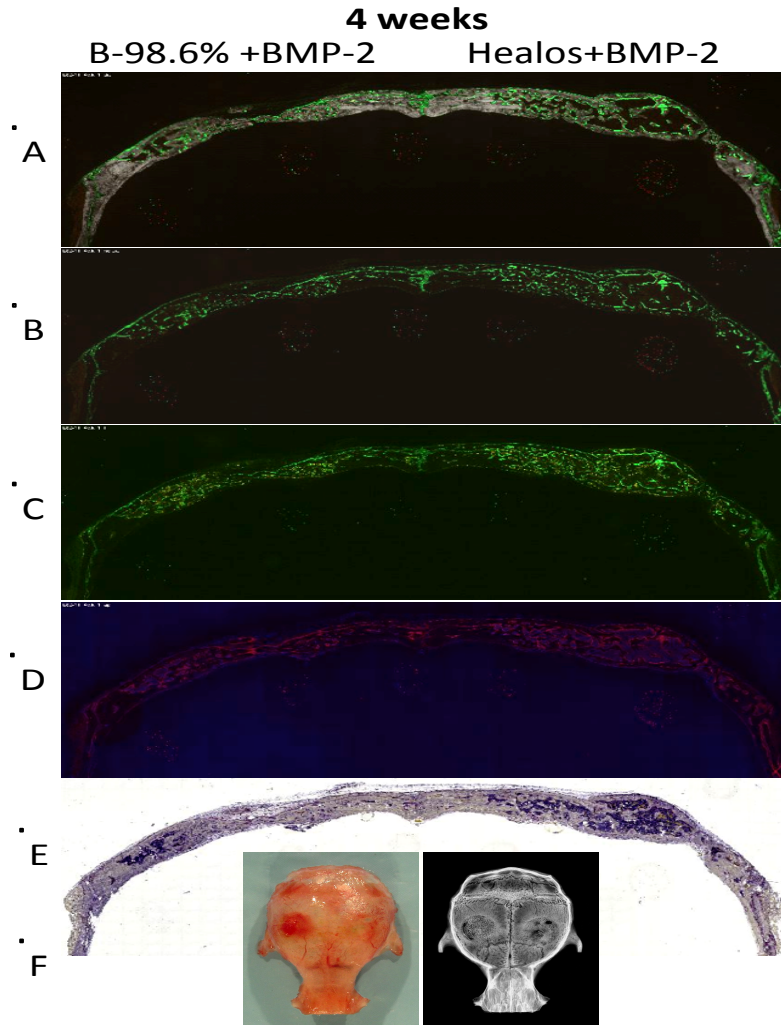


Figure 11. A) Fluorescent images showing mineralized bone; (B) shows image A without the mineral content C) TRAP staining indicating the osteoclast activity D) Alkaline phosphatase staining indicated osteoblast activity in the region; (E) H&E stained histological section F) x-ray & photographs of mouse calvaria post implantation

Figure 11 shows the feasibility of using chitosan as a BMP-2 delivery vehicle to increase bone regeneration using a parietal bone model. The photograph (panel F) shows the feasibility of retaining the gel with regenerated bone at the defect site at 4 weeks post implantation. The Panel A shows the fluorescent images of the slides with mineralized host bone. The chitosan thermogel or healos both with rhBMP-2 did promote significant bone formation. Panel B shows the same section without the mineralized bone content to clearly demonstrate significant infiltration of host osteoblast cells as evident from intense green fluorescence throughout the

defect site. Panel C shows the trap staining of the slides to evaluate the osteoclast activity. Not much trap activity was observed at 4 weeks in these samples. Panel D shows the alkaline phosphatase activity. Consistent with panel B observation, significant alkaline phosphatase activity was observed in the implant site 4 weeks post implantation. Panel E shows the H&E stained sections showing the overall cellular activity within and around the cells.

Specific aim 3; Task I & II: Implantation of various compositions in critical size femoral defects in reporter mice and following the cellular response via histology, histomorphometry, and immunohistochemistry and quality and quantity of regenerated bone via microCT and biomechanical testing. For donor cell isolation, the animals are currently been bred and will be available for use in 2-3 months.

Methods

Surgical procedure for segmental bone defect model

A new model for evaluation of bone regeneration using injectable compositions was developed where a segmental defect was generated in the femur (Figure 12 a). Briefly, the mouse was anesthetized and then the hind legs were shaved for fur, and swabbed with alcohol. A ~1cm incision was made on the skin through the muscle to expose the femur. A segmental defect was generated using a micro-saw and a plastic external fixation rod was used to maintain an open space of 2-3 mm and to prevent rotational instability of the bone defect. The figure shows the representative images of the surgical procedure. The chitosan gels were injected into the defect space and then the defect was closed by layer-by-layer suturing. The progress of bone healing was tracked by x-ray. B-83-87.9% gels with different concentration of BMP-2 (1 μ g and 2 μ g) were tested with respect to control (empty defect). The study is in progress.

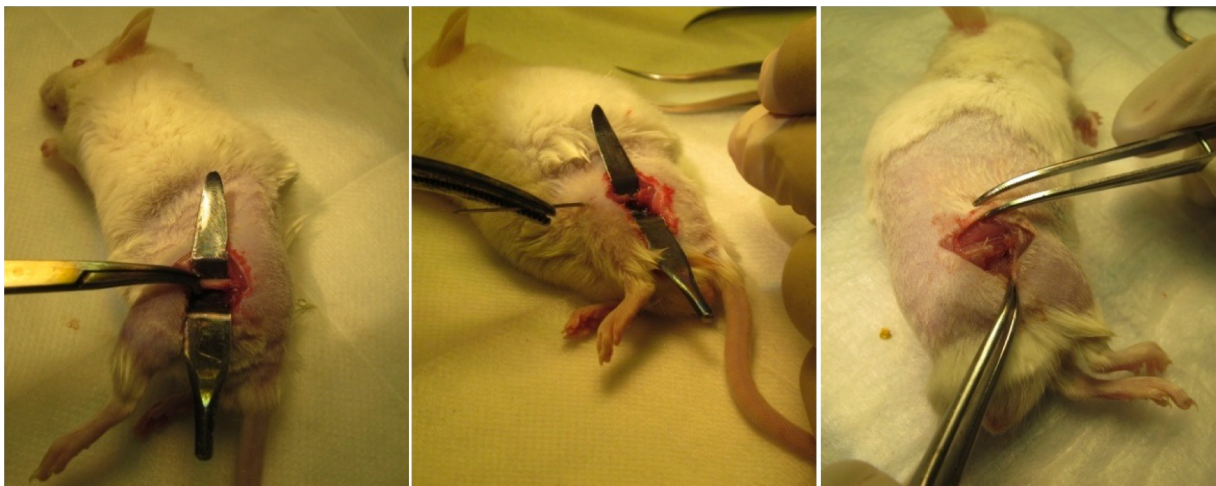


Figure 12a. A series of photographs illustrating various steps of surgical procedure used for generation of critical sized bone defect using segmental bone defect model

Results

A better and clinically relevant model was developed for testing the bone forming capability of injectable chitosan compositions with and without BMP and donor cells, using a long bone (femur) segmental bone defect model. Figure 12b shows the preliminary x-ray images showing various stages of healing post-implantation with respect to empty defect (control). The studies are currently in progress.

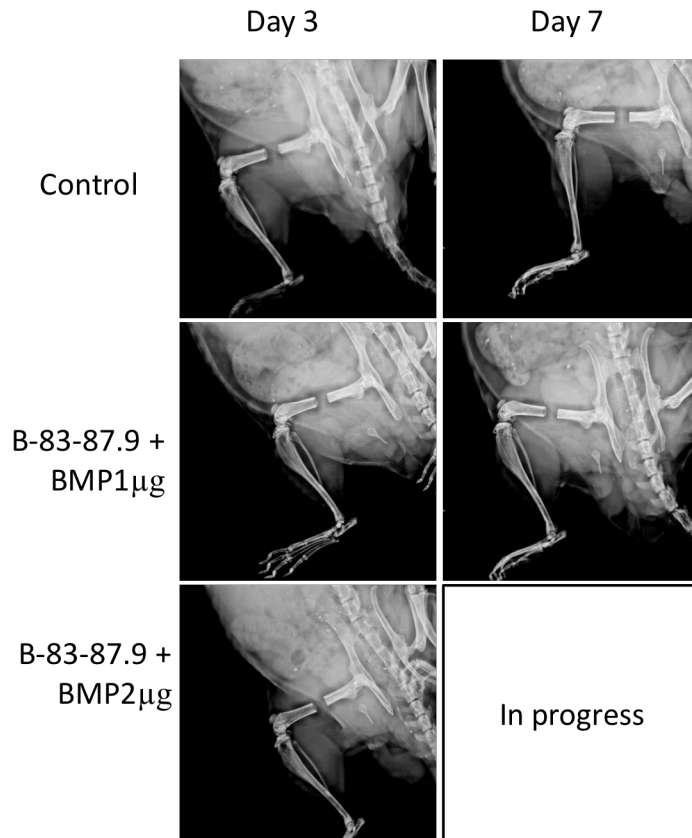


Figure 12b. X-ray images showing segmental bone defect healing at various time points post implantation

FUTUTE DIRECTIONS

- Study the effect of chitosan matrices in supporting bone regeneration in combination with rhMP-2 and MSCs, using mouse calvarial defect using histological and biomechanical evaluation. The study is in progress
- Study the effect of optimized chitosan matrices in combination with rhMP-2 and MSCs, in critical size bone defect using long bone segmental bone defect model. The study is in progress
- To increase the regenerative ability of chitosan gel by incorporating biological entities along the chitosan back bone

KEY RESEARCH ACCOMPLISHMENTS

1. Analyzed the effect of chitosans of different degree of deacetylation on cell morphology and cytokine release profile in mouse macrophages
2. Demonstrated that the degree of deacetylation can significantly affect macrophage morphology and cytokine profile.
3. Studied the effect of chitosan chemistry on host cell response in calvarial bone defect model
4. Identified the chitosan deacetylation range for further bone healing studies
5. Optimized the method of chitosan gel implantation to retain the gel at surgical site in mouse calvarial bone defect model
6. Performed preliminary study showing the effect of BMP-2 addition on bone regeneration
7. Optimized the immunohistochemical techniques to follow the effect of chitosan degree of deacetylation on macrophage cells in vivo and performed a preliminary study evaluating the same.
8. Optimized the long bone segmental defect model for evaluating the bone regeneration capability of injectable chitosan compositions in vivo

REPORTABLE OUTCOMES

Book chapter:

1. Singh H, Nair LS. Injectable in situ gelling hydrogels as Biomaterials., Handbook of Integrated Biomaterials for Medical Applications. Ramalingam M, Tiwari A, Ramakrishna S, Kobayashi H (Eds). VBRI press; pp. 359-396 (2012)

Journal Articles:

1. Cui Z, Wright L, Guzzo R, Freeman JW, Drissi HM, Nair LS. PDLA/PCL nanofiber-thermogelling chitosan gel composite scaffolds for osteochondral tissue regeneration in a rat model. J Bioactive Compatible Polymers (in press)
2. Foley PL, Ulery B, Kan H, Burks M, Cui Z, Wu Q, Nair LS, Laurencin CT. Sustained neural blockade via chitosan thermogel delivery of ropivacaine nanoparticles. J Controlled Delivery (Submitted)

3. McLaughlin S, Cui Z, Starnes T, Laurencin CT, Kan H, Wu Q, Nair LS. Injectable thermogelling chitosan for the local delivery of bone morphogenetic protein. *Journal of Material Science: Materials in Medicine* (2012) Jun 3. [Epub ahead of print]

Abstracts and proceedings:

1. James EN, Ulery B, Nair LS. Biomaterials for Therapeutic Gene Delivery. *American Ceramic Society's Ceramic Transactions Proceedings*, Vol: 237: Biomaterials Science-Processing, Properties and Applications II. Narayan R, Bose S, Bandyopadhyay (Eds) 2012 (in press)
2. Cui Z, Maye P, Adams D, Rowe DW, Nair LS. Evaluation of cellular response to chitosan thermogels using bone cell lineage reporter mouse calvarial model. *The Advanced Technology Applications for Combat Casualty Care (ATACCC)*, Fort Lauderdale, FL, August (2012)

CONCLUSIONS

The study demonstrated the ability of chitosans with different degree of deacetylation to affect macrophage morphology, viability and modulate cytokine profile. We developed the optimal method to introduce very small amounts of injectable solution and retain them on a mouse parietal bone using a pre-gelling formulation. We investigated chitosans with two different degree of deacetylations and followed the ability of the gel to support host cell infiltration. Both the chitosans studied were found to be compatible, however the chitosan gel with higher degree of deacetylation showed more cellular infiltration along with macrophage infiltration. We therefore decided to use chitosan gels with degree of deacetylation above 85% for the future studies. We also tested the feasibility of using chitosan gel to deliver rhBMP-2 to the parietal bone defect. The samples with rhBMP-2 showed significant bone formation showing it a conducive environment to support bone formation. We also performed pilot studies to develop long bone defect model in mice and optimized the feasibility of injecting and retaining chitosan at the defect site.

References:

1. Lee HS, Stachelek SJ, Tomczyk N, Finley MJ, Composto RJ, Eckmann DM. Correlating macrophage morphology and cytokine production resulting from biomaterial contact. *J Biomed Mater Res A*. 2012 Jul 30. doi: 10.1002/jbm.a.34309. [Epub ahead of print]