Effects of a novel hydrogel on equine bone healing: A pilot study

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Summary
Objective: To examine the efficacy and biocompatibility of a thiolated gelatin-thiolated carboxymethyl hyaluronan (CMHA-SGX) sponge as an osteoconductive device in an equine second and fourth metacarpal bone defect model.

Methods: Seven millimetre segmental osteotomies were created bilaterally in the second and fourth metacarpal bones of four horses. The left and right metacarpal defects were randomly assigned to (1) be filled with CMHA-SGX sponge (treated) or (2) were left unfilled (control). The duration of the study was nine weeks. Bone healing was evaluated using serial radiology, as well as histologically and histomorphometrically. Data were analyzed using an analysis of variance (ANOVA). The level of significance was p < 0.05.

Results: Serial radiographic evaluation revealed improved healing in the treated compared to the control defects at weeks eight and nine (p = 0.02). This finding was not corroborated histologically. Histomorphometry did not reveal any significant differences in healing between experimental groups. The CMHA-SGX sponge did not inhibit bone formation, induce local inflammation or lead to surgical site infection.

Clinical significance: While further optimization to improve osteoconductive properties should be considered, the CMHA-SGX sponge appears to be a biocompatible orthopaedic implant and its use as a carrier for osteogenic proteins warrants further investigation.

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Introduction
The emerging use of local osteoinductive proteins in human and veterinary orthopaedic repair has necessitated the development of novel carrier-based delivery systems for these substances. The beneficial effects of osteogenic proteins on bone repair in small animal models have not been observed in large animals or non-human primates without the addition of a carrier (1). This interspecies difference may be attributed to a slower bone formation rate and a smaller pool of available responsive stem cells in large animals (1). Effective use of exogenously administered osteogenic proteins in these species, therefore, necessitates the use of a carrier which enables a longer retention time and higher concentration of proteins at the fracture site (2). An ideal carrier used for these purposes should have a neutral or beneficial effect on bone healing, not interfere with the osteoinductive properties of the osteogenic protein, possess the optimal structure and porosity to maximize osteoconduction, be non-immunogenic, non-toxic and non-carcinogenic and be easy to handle and sterilize and suitable for commercial production (1–5).

Substances which have previously been considered as carrier vehicles for osteogenic proteins include naturally derived polymers (i.e. demineralized bone matrix [DBM], collagen or hyaluronan), synthetic polymers (i.e. poly-L-lactide or poly-L-co-D,L-lactide membranes) or ceramics (tricalcium phosphate or hydroxyapatite). Recently Liu et al. reported improved healing of rat femoral cortical bone defects implanted with a novel synthetic extracellular matrix comprised of a thiolated gelatin (Gtn-S) and a thiolated carboxymethyl hyaluronan (CMHA-S) (CMHA-SGX, previously termed Carbylan™-GSX)(5). Hydrogel and sponge forms of CMHA-SGX without an osteogenic protein were shown to improve bone healing in this model; however, the best results were observed when the sponge form of CMHA-SGX was impregnated with DBM powder (5). These results suggest that the sponge form of CMHA-SGX may be a good carrier for DBM or other bone graft substitutes.

The purpose of our study was to examine the efficacy and biocompatibility of the sponge form of CMHA-SGX as a device for accelerating bone healing in an equine, non-critical sized second and fourth metacarpal bone defect model (6, 7). We hypothesized that (1) the CMHA-SGX sponges would be non-immunogenic and that there would be no difference in the amount of local inflammation observed in bone defects filled with CMHA-SGX sponges compared to untreated bone defects; (2) there would be no difference in postoperative complications between bone defects filled with CMHA-SGX sponges and untreated bone defects; and (3) bone defects filled with CMHA-SGX sponges would demonstrate accelerated rates of healing com-
pared to untreated bone defects as judged by serial radiographic monitoring and terminal histological and histomorphometric analysis.

Materials and methods

Horses
Four mature horses (500–590 kg, 3–10 years old) were used. A physical examination was performed and four standard digital radiographic projections (dorso-palmar, lateromedial, dorsolateral-palmaromedial oblique, and dorsomedial-palmarolateral oblique) were obtained of the metacarpal region of both forelimbs prior to entry into the study. Horses that were systemically ill or with lameness observable at a walk, and horses with abnormalities of the proximal and mid-metacarpal region were excluded. Prior to beginning the study, horses were given a seven-day acclimatization period and were vaccinated and dewormed. Horses were housed in individual box stalls and fed hay and water *ad libitum*. The protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee at the University of Pennsylvania.

Study design

Seven millimetre segmental ostectomies of both the left and right second and fourth metacarpal bones were performed on four horses. The study design is outlined in Fig. 1. Numbers (1 to 4) were randomly assigned to the four horses.

CMHA-SGX sponges

Sponges were made as previously described (5). Briefly, a 2.5% (w/v) solution of CMHA-S and a 3% (w/v) solution of Gtn-S were mixed in a 1:1 volumetric ratio. The resulting solution was then mixed in a 4:1 volumetric ratio with a 4% (w/v) solution of polyethylene glycol dia-crylate (PEGDA; MW 3350) to create the CMHA-SGX solution. CMHA-S, Gtn-S, and PEGDA were all synthesized according to standard protocols (5, 8). The CMHA-SGX solution was then poured into a 30 mm diameter x 10 mm thick mould and allowed to crosslink at room temperature, exposed to air for two hours. The resultant cross-linked CMHA-SGX hydrogel was frozen at –80 °C and lyophilized to create the CMHA-SGX sponge. Sponges were placed in medical pouches *b* and sterilized using gas plasma *c*.

Perioperative protocol

A preoperative physical examination, haematology and determination of plasma creatinine concentration were performed. Procaine penicillin Gd (22,000 IU/kg IM q 12 h for 3 doses) and phenylbutazone *d* (4.4 mg/kg IV intra-operatively then 2.2 mg/kg PO q 12 h for 5 days) were administered.

General anaesthesia

Horses were sedated with xylazine hydrochloride (0.3–0.6 mg/kg IV) and butorphanol tartrate (0.01–0.02 mg/kg IV). Anaesthesia was induced with guaifenesin (15–30 mg/kg IV), diazepam (0.1 mg/kg IV) and ketamine hydrochloride (2.2 to 2.5 mg/kg IV) and maintained using isoflurane in oxygen.

Segmental second and fourth metacarpal ostectomy

After preparation for aseptic surgery, a 6 cm longitudinal incision was created through the skin and subcutaneous tissue over the middle third of the intended metacarpal bone. The incision was extended through the periosteum, which was elevated away from the bone and resected with Metzenbaum scissors. A sterile ruler was placed between the palmar aspect of the intended bone and the body of the suspensory ligament to prevent ligament damage. An oscillating saw with two parallel blades spaced 5 mm apart was then used to create...
a 7 mm ostectomy (Fig. 2). The interosseous attachments between the osteotomized bone and the third metacarpal bone were severed using an osteotome and the 5 mm segment of bone was removed. Any remaining bone fragments or sharp edges were removed from the ostectomy site using curettage and the site was lavaged with saline. The ostectomy site was filled with a 5 mm CMHA-SGX sponge (group T; Fig. 3) or was left empty (group C). The incision was routinely closed in two layers and a padded compression bandage was applied for recovery.

Postoperative care

A physical examination was performed every 12 hours and the horses were monitored for signs of lameness. Horses were maintained in a padded compression bandage for 12 days following surgery; bandages were changed at least every three days and the surgical site was monitored for swelling and signs of infection, drainage or dehiscence. Sutures were removed 12 days after surgery.

Radiographic evaluation

Digital radiographs were obtained of each limb 24 hours after surgery and then every two weeks for eight weeks. A final set of radiographs was obtained on day 63 immediately prior to euthanasia. Radiographic projections included a dorsopalmar, a dorsolateral-palmaromedial oblique and a dorsomedial-palmarolateral oblique of each metacarpal region. The radiographs were independently graded by three of the authors (JMC, LLS, ML) who were unaware of the treatment group assignment. Radiographic grade was based on a subjective assessment of the percentage of each defect which was filled with bone and the degree of proliferative change around each defect. In order to best estimate the percentage of the defect which was filled with bone, each ostectomy site was divided into four zones (Fig. 4), each of which was assigned a score of 0%, 25%, 50%, 75% or 100% bone fill. To determine radiographic assessment of healing, each zone was examined separately in addition to a total radiographic grade which was equal to the mean of the scores from the four zones.

Euthanasia and tissue harvest

Horses were euthanatized using pentobarbital (88 mg/kg IV) on postoperative day 63. Immediately following euthanasia, the front limbs were disarticulated at the carpus for tissue harvest. Each ostectomy and its adjacent region of the third metacarpal bone were harvested for histomorphometric analysis and histological evaluation. All samples were fixed in 10% neutral buffered formalin, dehydrated and infiltrated with and embedded in methyl methacrylate without decalcification. Samples were cut in an oblique plane to 140 to 180 μm sections and then polished to 100 μm thickness using a diamond band saw and grinding system.

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Fig. 2
Creation of the 7 mm segmental ostectomy in the left, 2nd metacarpal bone of Horse 4. The completed bone defect is shown in the upper right corner.

Fig. 3
Placement of the CMHA-SGX sponge into the bone defect.

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EXAKT Technologies: Oklahoma City, OK, USA
Histology

Non-decalcified, 100 μm thick longitudinal sections through the ostectomy site were stained with haematoxylin and eosin for evaluation using light microscopy. A single representative section from each defect site was examined and the extent and character of the callus was documented. Specifically, the degree of osseous callus formation (graded on a scale of complete, near complete, partial or absent), the percentage of ossified tissue within the callus, the percentage of lamellar versus woven bone within the callus, and the percentage of trabecular versus compact bone within the callus were examined. In addition, the presence or absence of cartilage within the callus, the presence or absence of inflammatory cells at the ostectomy site, and the degree of osteopenia of the bone distal to the ostectomy site were also assessed. Degree of osteopenia was assessed on a scale of 0 to 3 where 0 represented normal bone density and 3 represented severe loss of bone density. All histological assessments were performed by a board certified veterinary pathologist (JE) who was unaware of the experimental group assignments.

Histomorphometric analysis

For quantitative assessment of the size of the callus, haematoxylin and eosin stained sections were examined under light microscopy at 2X magniﬁcation using raw scale units. These measurements were performed by a board certified veterinary pathologist (JE) and included the percentage of the longitudinal defect that was bridged with callus, the percentage of the transverse defect that was bridged with callus, the total area of new bone and the percentage of the total defect that was filled with bone. Digital imaging software was used to determine the density of the new bone. For this histomorphometric analysis, haematoxylin and eosin stained sections were examined under light microscopy at 4X magnification. The image was then captured using a digital camera attached to the microscope and displayed on the computer monitor. Imaging software was used to digitally determine the percentage of each defect which was filled with bone as well as the bone density (extrapolated measurement based on a uniform slide width and stain.

Fig. 4 Zones used for radiographic grading.

Fig. 5 Group T (treated; filled with CMHA-SGX sponge) defect: Radiographic progression of healing. MCIV = Fourth metacarpal bone.
ing intensity) within the defect. All measurements obtained using imaging software were made in triplicate by one of the authors (JMC) who was unaware of the experimental group assignment.

Statistical analysis

Using a commercially available software programme\(^1\) continuous data were analyzed using a mixed model analysis of variance (PROC MIXED). The dependent variables included radiographic scores and histomorphometric data (percentage of the ostectomy site filled with new bone, density of the new bone within the ostectomy site, percentage of bridging callus within the defect, area of new bone formation, callus size, percentage of lamellar versus woven bone, percentage of compact versus trabecular bone, and percentage of ossified bone within the defect). The random variable was the horse. The class variables were the limb (left, right), the bone (metacarpus II, IV), time (week 2, 4, 6, 8 postoperatively), and treatment group (treated, control). The interactions between the class variables were included in the model. When the interactions were not significant, they were excluded from the final model. A post-hoc Tukey’s adjustment was used for multiple comparisons. Inter-observer agreement for radiographic scoring was assessed using a three way Pearson correlation\(^1\) (PROC CORR). Categorical data were analyzed using a Fisher’s Exact test\(^1\) (PROC FREQ). The level of significance for all statistical analyses was \(p < 0.05\).

Results

Postoperative evaluation

All horses recovered well from surgery. Horses were sound at a walk 24 hours postoperatively and remained free of lameness for the duration of the study. Minimal swelling was observed at the surgery sites. All incisions healed without complication.

Radiographic evaluation

There was a significant increase in the percentage of bone filling the defect in both groups within all zones (A-D) over time (\(p = 0.005\)) (Fig. 5 and 6). Defects in the treated group had a higher percentage of bone in zones A, B, and D at weeks eight and nine postoperatively compared to defects in the control group; however, this difference was statistically significant only in zone D (\(p = 0.005\) at 8 weeks and \(p = 0.01\) at 9 weeks) (Table 1).

A three-way Pearson’s correlation revealed excellent agreement between the three independent observers with regards to the semiquantitative radiographic scoring (\(r = 0.78\) to \(0.88\), \(p < 0.001\)). Immediate postoperative radiographs showed varying

\(^1\) Statistical Analysis System: SAS Institute Inc., Cary, NC, USA
amounts of bone on the axial aspect of the defect and some slight scoring of the third metacarpal bone; however, there was no significant difference between experimental groups.

**Histology**

Varying degrees of intramembranous bone healing was observed in all defects. There were no islands of cartilage in non-decalcified sections stained with haematoxylin and eosin. New bone filling the defects varied in maturity in terms of the amount of lamellar versus woven bone, the amount of compact versus trabecular bone, and the degree of ossification (Table 2). A trend towards a higher percentage of lamellar and compact bone in group C defects compared to group T defects which had a higher percentage of woven and trabecular bone was noted (Fig. 7 and 8); however, this difference was not significant (p = 0.07 for both measurements). Minimal inflammation was observed within the bone defects. No residual implant was observed within the group T defects. Mild to moderate osteopenia was observed distal to the defect in some bones, but this was not different between experimental groups. Degree of callus formation ranged from complete (8 defects) to near complete (4 defects) to partial (4 defects). The bridging callus formation was not significantly different between experimental groups.

**Histomorphometric analysis**

There were not any significant differences between experimental groups in the percentage of each defect that was filled with bone (C: 81 ± 19 and T: 82 ± 16%) or the density of the bone filling each defect (C: 9.8 ± 2.0 and T: 8.6 ± 1.7 pixels/cm²). There were not any significant differences between experimental groups when comparing the longitudinal or transverse percentage of the defect bridged, the area of new bone or the callus size.

**Discussion**

The purpose of this study was to determine the effect of the CMHA-SGX sponge on healing of segmental ostectomy sites in equine second and fourth metacarpal bones. We hypothesized that (1) there would be no difference in the amount of local inflammation observed in bone defects filled with CMHA-SGX sponges compared to untreated bone defects; (2) there would be no difference in postoperative complications between bone defects filled with CMHA-SGX sponges and untreated bone defects; and (3) bone defects filled with CMHA-SGX sponges would demonstrate accelerated rates of healing compared to untreated bone defects as judged by serial radiographic monitoring and terminal histological and histomorphometric analysis. The findings of this study support our first two hypotheses but fail to provide sufficient evidence to support the claim that the CMHA-SGX sponge alone accelerates the rate of bone healing in this model.

Serial sets of radiographs were used to evaluate progressive healing of the treated and control bone defects over time. A significant difference in the radiographic healing of the bone defects in the eight experimental groups was not observed until eight weeks after surgery. At that time, although all zones showed trends toward more tissue in the treated than the control defects, zone D was judged to contain significantly more mineralized tissue in the treated defects than in the untreated con-
control sites. Furthermore, while the radiographic data suggests a modest beneficial effect of the CMHA-SGX sponge on bone healing, this finding was not substantiated by the histological evaluation or histomorphometric analysis. It is not clear why the radiographic impression of significantly increased tissue filling in the treated defects compared to controls was unsupported by histomorphometric analysis. This discrepancy in the analyses might be explained in that zone D was the only zone in which significant differences were observed radiographically and the histomorphometric analysis was not performed on a zone-by-zone basis.

The extent of the beneficial effect on bone healing previously observed when CMHA-SGX sponges were implanted in femoral defects in rats for eight weeks was not observed in our study (5). This finding may suggest that the chemical or physical properties of the CMHA-SGX sponge may not be optimized for osteoconduction in equine bone. For instance, these sponges are based on a mixture of a modified hyaluronan and a modified gelatin. Modified hyaluronan alone exhibits poor cell adhesive qualities, rendering it particularly useful for some applications such as post-surgical adhesion prevention (9). However for applications where cell adhesion and cell infiltration are desired, a cell adhesive protein or peptide is required. Arginine-glycine-aspartic acid (RGD), a ubiquitous cell adhesion ligand, fibronectin fragments and gelatin have all been incorporated into these materials to promote cell adhesion. Although this study utilized the same cell adhesion component (Gln-S) as previously used for healing bone defects in rats, this component or amount thereof, may not be optimized for equine (or other large animal) bone healing (10–12).

The pore size, shape and surface roughness of a synthetic matrix can also affect its cellular adhesion properties as well as the local cellular phenotype and rate of proliferation (4). Although the 350 μm pore diameter of the CMHA-SGX sponge is well within the recommended 200 to 400 μm range, it is possible that the physical characteristics of the implant could be improved to increase the osteoconductive properties of this implant in equine bone (4, 5, 13). A similar effect, observed in healing of equine cortical defects implanted with 2 to 4 mm particles of DBM, was proposed to have resulted from a potential space-occupying effect associated with the physical (geometric) property of the DBM which may have inhibited the in-growth of new bone (14).

Limitations with the current study include the small sample size and subsequently low statistical power to conclude that there was no difference between experimental groups. The study, however, was sufficiently powered to detect a difference in defect filling between experimental groups at eight and nine weeks postoperatively. A sample size greater than 100 horses per group would have been required to detect a difference in zone D filling between experimental groups at six weeks. Despite our efforts during surgery to ensure all bone was removed from the defect, there were small spicules of bone remaining in the defect postoperatively in zones A and B for both groups. These spicules of bone were included in the percentage radiographic filling of bone defects on day 1 postoperatively (Table 1); however, there was no significant difference between groups at this time point.

Based on gross and histological evaluation, the CMHA-SGX did not delay new bone formation, induce a local pro-inflammatory effect or lead to surgical site infection. Although a modest beneficial effect was observed radiographically in the CMHA-SGX treated bones, this observation was not supported histologically. While further optimization of this material
to improve its osteoconductive properties in large animal bone should be considered, no negative effects were observed following implantation of the CMHA-SGX sponge in equine bone. In its current form, the CMHA-SGX sponge should be considered a biocompatible orthopaedic implant and its use as a carrier vehicle for DBM or osteogenic proteins warrants further investigation.

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Conflict of interest
The authors have no conflict of interest and have no financial interest in SentrX Animal Care.

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