**Title:** Enhanced bone healing using collagen-hydroxyapatite scaffold implantation in the treatment of a large multiloculated mandibular aneurysmal bone cyst in a Thoroughbred filly

**Running Head:** Collagen-HA scaffold shows enhanced bone healing in a clinical case study

**Authors:**

* Florent David¹,², DVM, MSc, Dipl. ACVS/ECVS, ECVDI Assoc., Dipl. ACVSMR

* Tanya J. Levingstone³,⁴,⁵, MSc, BEng, PhD

Wilfried Schneeweiss¹,⁶, MVM, Dr. med. Vet.

Marie de Swarte¹, DVM

Hanne Jahns¹, MVM, PhD, Dipl. ECVP

John Gleeson³,⁴,⁵,⁷, BA, BAI, MSc, PhD, MIEI

Fergal J. O’Brien³,⁴,⁵, BA, BAI, PhD, CEng, FIEI

* both authors contributed equally to the work

**Authors’ affiliations:**

¹ University College Dublin Veterinary Hospital, School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.
Mid-Atlantic Equine Medical Center, 40 Frontage Rd, Ringoes, NJ, 08551, the United States of America.

Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland.

Advanced Materials and Bioengineering Research (AMBER) Centre, RCSI & TCD, Dublin, Ireland.

Pferdeklinik Pegasus, Laaberstrasse 69, 2384 Breitenfurt, Austria.

SurgaColl Technologies Limited, Rubicon Centre, Cork Institute of Technology, Rossa Avenue, Bishopstown, Cork, Ireland.

**Corresponding Author:**
Prof. Fergal O’Brien, Associate Professor, Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.

Phone: +353 1 4022149  Fax: +353 1 402 2355

Email: fjobrien@rcsi.ie

**Place where this case was operated:**
Ethical approval and consent form:

As this was a true clinical case and not an experimental case, no ethical approval was required. Authorisation from the Irish Department of Agriculture, Food and the Marine was granted to use the collagen hydroxyapatite (CHA) bone graft substitute in this specific case. The horse was permanently stamped “Out of the Food Chain”. The owner signed a consent form discharging the University College Dublin Veterinary Hospital and the Royal College of Surgeons in Ireland from any legal responsibilities.

Conflict of interest:

Authors John P Gleeson and Fergal J O’Brien hold IP with a commercial product of related composition to the collagen-HA scaffolds used in this study. SurgaColl Technologies Limited provided partial financial support to the University College Dublin Veterinary Hospital, School of Veterinary Medicine, University College Dublin, to facilitate additional post-implantation clinical imaging of the animal.

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**Keywords:**

bone graft substitute, collagen-based scaffolds, equine, tissue engineering, mandibular aneurysmal bone cyst, Computed tomography
Abstract (Max 250 words)

An unmet need remains for a bone graft substitute material that is biocompatible, biodegradable and capable of promoting osteogenesis safely in vivo. The aim of this study was to investigate the use of a novel collagen-hydroxyapatite (CHA) bone graft substitute in the clinical treatment of a mandibular bone cyst in a young horse and to assess its potential to enhance repair of the affected bone. A 2 year old Thoroughbred filly, presenting with a multilobulated aneurysmal bone cyst was treated using the CHA scaffold. Post-operative clinical follow-up was carried out at 2 weeks and 3, 6 and 14 months. Cortical thickening in the affected area was observed from CT examination as early as 3 months post-surgery. At 14 months, reduced enlargement of the operated mandible was observed, with no fluid filled area. The expansile cavity was occupied by moderately dense mineralised tissue and fat and the compact bone was remodelled, with a clearer definition between cortex and medulla observed. This report demonstrates the successful application of the CHA scaffold material in the promotion of enhanced bone repair in this craniomaxillofacial indication and thus the potential of this material for translation to human applications.
1. Introduction

Segmental bone defects, occurring as a result of fractures, tumours, bone cysts and other diseases, remain a significant challenge for orthopaedic surgeons. Currently, the “gold standard” clinical approach involves the surgical harvesting of autograft tissue, taken from the patient's own body and subsequently re-implanted into the defect site. However, due to the limitations associated with autograft tissue, particularly in the treatment of large area defects, alternative solutions are required. While tissue-derived substitutes such as allografts and xenografts can offer practical advantages over autograft material (e.g. no need for additional surgery, “off the shelf” availability, size of graft material), their use is limited due to concerns over immune reactions and transfer of host diseases.

Focus has now moved to the development of bone graft substitutes and tissue engineered biomaterial scaffolds. Numerous materials are currently under development, with investigators working to optimise scaffold properties including biocompatibility, osteoinductivity, osteoconductivity, mechanical resilience, and functional resorption while minimizing inflammation and foreign body reaction (Szpalski et al., 2012). More recently, there has recently been a move towards the incorporation of cells, growth factors and cellular signalling molecules into these scaffold materials. In particular, the use of
growth factors for stimulating bone repair in challenging surgical cases has become popular since the identification of bone morphogenetic protein-2 (BMP-2) as an important growth factor in bone formation. Recombinant versions (rhBMP-2 Infuse, Medtronic, Inc (FDA, 2002) and rhBMP-7, (OP-1, Novo Noradisk (FDA, 2001)) have received FDA approval for specified surgical procedures, but initially successful results (Sciadini and Johnson 2000; Yasko et al., 1992) and subsequent human trials (Boden et al., 2000; McKay et al., 2002) have been called into question by numerous studies citing safety concerns (Garrett et al., 2010; Shields et al., 2006; Vaidya et al., 2009; Wong et al., 2008). In addition, such is the need for a viable alternative to autogenous bone that up to 85% of reported BMP-2 use was off-label (Services DoHaH, 2010), thus leading to the US Department of Health and Human Services calling for a review of current evidence on the safety of rhBMP-2 doses and applications (Services DoHaH, 2010). While this is concerning for orthopaedic surgeons, it simply means that there is still an enduring and unmet need for a bioactive, load-bearing tissue-engineering scaffold, which is biocompatible, biodegradable and capable of facilitating and promoting significant osteogenesis safely when implanted in vivo.

Research in the Tissue Engineering Research Group (TERG) in the Royal College of Surgeons in Ireland, has led to the development of a
biocompatible, biomimetic and highly porous (> 95%) collagen hydroxyapatite (CHA) composite scaffold (Gleeson et al., 2010). The CHA scaffold is fabricated by incorporating a ceramic hydroxyapatite particle phase into a collagen-based scaffold using a patented mixing process (O’Brien et al., 2007) to produce a highly porous scaffold with a composition optimised for bone repair. The osteoconductive properties of the scaffold have been demonstrated in vitro (Gleeson et al., 2010). Regenerative potential has been demonstrated in vivo in a rat calvarial defect (Gleeson et al., 2010) and in load bearing rabbit radial model (Lyons et al., 2014). Significantly, the CHA scaffold demonstrated comparable results to a collagen GAG (CG) scaffold loaded with BMP2 (Lyons et al., 2014). Further analysis has demonstrated that the positive healing response is due to the innate osteoinductivity of the scaffold as a result of the method of incorporation and presentation of HA within it (Murphy et al., 2014).

This study describes the use of this novel CHA bone graft substitute as a viable alternative to autogenous bone in the treatment of an aneurismatic bone cyst in the mandible of a 2 year old Thoroughbred filly. Aneurysmal bone cysts (ABCs) are rare bone lesions that can affect the axial and appendicular skeleton of young animals. The mandible is the most common location for ABCs in horse. In humans, the metaphysis of long bones, pelvis, and vertebral column are the
most commonly affected areas (Cottalorda et al., 2004). There is currently little consensus regarding treatment options for such bone lesions and their ultimate effectiveness. Curettage is commonly performed and is sufficient for inactive lesions. However, the extent of mandibular cortical thinning in this case raised significant concerns about the use of curettage and long-term stabilisation of the tissue through normal bone remodelling processes with pathological fracture of the mandible posing a significant risk to the patient (Ordidge et al., 2001). The aim of this study was to thus investigate the use of the CHA bone graft substitute in this craniomaxillofacial indication and to assess its potential to promote osteogenesis and cortical thickening of the affected mandibular compact bone.
2. Materials and Methods

2.1 Case Description

A 2 year old Thoroughbred filly presented to University College Dublin Veterinary Hospital with a large firm swelling of the right mandible of unknown duration. The whole horizontal mandibular ramus was enlarged, filling almost the entire intermandibular space (Fig. 1A). Although no overt pain was noted under palpation, the area was warm to the touch and the filly anticipated palpation. She was observed to drink, eat and chew hay, her body condition was 2/5 (Carroll et al., 1988). Routine haematology and serum biochemistry were carried out and were unremarkable.

Radiography of the right mandible revealed a multiloculated radiolucent expansible lesion with “soap bubble appearance” extending from the mental foramen to the rostral root of the second molar (M2; Triadan 410) (Fig. 1B). The compact bone was ventrally thinner than normal but no periosteal reaction was noted. Misalignment and distortion of the permanent teeth was noted, as well as a suspicion of lysis of the 4th premolar (PM4; Triadan 408) tooth bud. Computed tomography (CT) revealed a fluid (Hounsfield unit (HU) 20) expansible mass in the horizontal ramus of the right mandible (Fig. 1D). The expansile mass extended from the right mental foramen to right mandibular M3 (Triadan 411) tooth bud (Fig.
The mandible measured 5.5 cm at its maximal width (left mandible at the same level 2.5 cm for comparison) and the mass occupied 3/4 of the height of the mandible. The right mandibular compact bone was thinner than normal (1-3 mm compared to 1.3-4.8 mm on the left mandible at the same level). It was also noted that in some focal areas the cortex was perforated and the right mental foramen was enlarged. The permanent teeth were distorted (mainly PM3 (Triadan 407)) and/or displaced by the mass. Hypoplasia of the bud of PM4 (Triadan 408) was also noted. Intraoperative aspiration of the cystic fluid was performed and cytology revealed a non-septic inflammation (TP=54 g/L, WBC=0.16 x10⁹/L, mainly macrophages) with mild past and recent haemorrhage and mild benign osteoclast proliferation. A presumptive diagnosis of a multilobulated mandibular bone cyst with tooth displacement, distortion and hypoplasia was made.

2.2 Scaffold Fabrication

CHA scaffolds were fabricated using a previously described freeze-drying technique. Briefly, Type 1 collagen (Collagen Matrix Inc., NJ, USA), hydroxyapatite ((Captal 'R' Reactor Powder, Plasma Biotal, UK) and a 0.5M acetic acid solution were combined using a patented blending protocol [13, 14]. The resultant CHA suspension was pipetted into stainless-steel trays (internal dimensions - 60mm x
60mm; 18ml CHA solution per tray) and freeze-dried (Virtis Genesis 25EL, Biopharma, Winchester, UK) at a constant cooling rate of 1 °C/min to a final freezing temperature of -40°C ((Gleeson et al., 2010; O’Brien et al., 2007). Following freeze-drying, dehydrothermal (DHT) treatment was carried out at a temperature of 105°C under a vacuum of 0.05 bar for 24 hours (Vacucell 22; MMM, Germany). Scaffolds were then chemically cross-linked for 2 hours at room temperature with 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC)/ N-Hydroxysuccinimide (NHS) (Sigma-Aldrich, Arklow, Ireland) at a concentration of 6 mM EDAC per gram of collagen and a 5:2 molar ratio of EDAC:NHS (Gleeson et al., 2010).

2.3 Surgical Procedure
The mare was anesthetised and positioned in dorsal recumbency. A ventromedial approach to the enlarged horizontal ramus was performed via a 20 cm long skin incision. The poorly adherent periosteum was reflected and the ventral surface of the bone examined. Black discolorations spots were visible on the compact bone surface. Two osteal windows (8 x 3 cm and 6 x 3 cm) separated by a 2 cm wide bridge were created using an oscillating saw to give access to the multilobulated cyst (Fig. 2B). A yellow serohaemorrhagic fluid was aspirated from one of the cysts (Fig. 2A). Suction was used to aspirate the remaining cystic fluid. The cyst
lining and bone spikes adherent to the dorsal aspect of the bone flaps were curetted and flushed (Fig. 2C). Six 2 mm holes were drilled in the corner/edge of the flaps and parent bone to enable flap re-apposition at the end of surgery. The flaps were preserved in swabs impregnated with saline and autologous venous blood and disposed in a sterile kidney dish.

Several connected cystic structures were visible within the mandible cavity (Fig. 2B). With assistance from the CT images, the cystic cavity was debrided using a curette. The mandibular nerve was identified and the debridement and curettage was initiated rostral to PM2 (Triadan 406). Any cystic and abnormal bone material was removed. The bud of PM2 was difficult to differentiate from the cystic tissue and was also debrided to remove any suspicious material. The debridement was then continued around PM3 (407), PM4 (408), M1 (409) and M2 (410). The cavity volume was estimated by filling with saline and found to be 240-250 ml in volume (Fig. 2C). The cavity was then washed with saline twice and all the fluid suctioned.

As a risk of traumatic/pathologic fracture was considered high on this case, CHA scaffold sheets, measuring 5 x 60 x 60 mm in dimension were placed into the defect site to encourage rapid bone healing. The scaffolds were positioned along the internal walls of the mandible
with 5 sheets inserted in total. Two CHA sheets were inserted (flat) on the medial side, one (flat) on the lateral side, one caudally (rolled) and one rostrally (rolled) in the cavity (Fig. 2D). The bone flaps were sutured in place to the parent bone using USP 2-0 polydioxanone suture material passed through drilled holes. The surface of the bone was then flushed with gentamicin (500 mg) and the periosteum sutured with polyglecaprone USP 2-0. A gentamicin (500mg) flush was repeated and the skin apposed using skin staples and a protective bandage was placed for recovery. Using a rope-assisted recovery system the mare recovered from anaesthesia uneventfully.

2.4 Post-operative Assessment

Post-operative clinical re-evaluation was performed at 2 weeks and 3, 6 and 14 months. CT examinations were performed at 3 and 14 months post surgery. In order to evaluate the remodelling of the right mandible following treatment, mandible bone thickness, and mandible cavity area and volume measurements were carried out using the OsiriX HD 4.0 software (Pixmio, Geneva, Switzerland). Measurements at each time point are reported as a % size difference relative to the left mandible (normal), thus accounting for any normal anatomical changes resulting from growth of the animal during the study. The cavity area measurements in each case were compared at the widest point of the mandible.
3. Results

3.1 Histopathology Results
Tissue samples (cystic material and material coming from PM2 (406)) harvested during surgery were sent for histopathology (Fig. 3A). The features of these samples were consistent with a multiloculated aneurysmal bone cyst. The dental material submitted was consistent with normal tooth root material.

3.2 Post-operative Outcome
Post-operative evaluation at 2 weeks revealed a fully healed surgical wound. At 3 months post-surgery, the operated mandible appeared subjectively less enlarged and the oral examination was within normal limits. A CT examination revealed that most of the expansile cavity was filled by moderately dense mineralised tissue (HU 200-300), with a few remaining fluid filled areas (HU 10) still apparent (Fig. 3C). The right mandible measured 5.6 cm at its maximal width (left mandible 2.4 cm). The compact bone was continuous and was generally thicker than previously described (1.2-10.7 mm compared to 1.9-7.2 mm at the same levels on the left mandible). Most of the tooth buds surrounding the cyst had grown but were still smaller compared to the opposite side. Their displacement and distortion was less severe than pre-surgery. The right mandibular PM2 tooth bud
showed signs of resorption of its roots. The area at the widest point of the mandible was reduced compared to pre-operative measurements, although the volume was slightly increased, most likely due to growth of the animal (Fig. 4).

At 6 months post-surgery the horse was in training and no problems with her jaw, masticatory function or bite acceptance were recorded. At 14 months post-surgery, the operated mandible appeared subjectively less enlarged than at 3 months. A non-painful bony prominence was noted on the ventral-lateral-rostral aspect of the right mandible. On oral examination the decidual PM3 (Triadan 807) on the right mandible was missing while it was still firmly attached on the left mandibular arcade. Eruption of the permanent PM3 (Triadan 407) could be palpated in the gap between PM2 (Triadan 406) and PM4 (408). A repeat CT examination revealed the right mandible measured 4.5 cm at its maximal width (left mandible 2.7 cm). There was no fluid filled area and the expansile cavity was occupied by moderately dense mineralised tissue (HU 150-300) and fat (HU 20-100) (Fig. 5). The compact bone of the right mandible was remodelled and a clearer definition between cortex and medulla was noted. The cortex was thinner than at 3 months post-surgery (1-2.4 mm compared to 0.7-4.7 mm at the same levels on the left mandible). The cavity area and volume measurements showed the
right mandible to be less enlarged than prior to surgery (Fig. 4). On the right side PM2 (Triadan 406) appeared to have erupted correctly but this tooth was significantly shorter than 306 (3.2 cm versus 7.4 cm). PM3 (Triadan 407) seemed to have just erupted. This tooth was still slightly distorted with its root pointing laterally, deforming the right mandible externally. Both PM4 (Triadan 408 and 308) presented the same length and were covered by their dental caps. The molars were symmetric between left and right and normal in appearance.

4. Discussion

The study demonstrates the successful clinical use of a collagen-hydroxyapatite bone graft substitute for the treatment of an equine craniomaxillofacial bone cyst. The CHA scaffold applied in this case has been designed to address a major unmet need, for a bone graft substitute material that is biocompatible, biodegradable and capable of promoting osteogenesis safely in vivo. Follow-up at 3 and 14 months post-implantation revealed reduced enlargement of the operated mandible, initial thickening of the compact bone with no fluid filled area, and later remodelling of the compact bone with a clearer definition between cortex and medulla. The results show the potential of the scaffold to promote osteogenesis and cortical thickening of the affected mandibular compact bone.
An aneurysmal bone cyst is a reasonably rare condition occurring in animals (Thompson et al., 2007; Bryant et al., 2012) and humans (Cottalorda et al., 2004). Many hypotheses have been proposed to explain the etiology and pathogenesis of aneurysmal bone cysts (Jaffe et al., 1942; Lichtenstein et al., 1957). One of the more commonly accepted ideas that increased venous pressure and a resultant dilation and rupture of the local vascular network could trigger onset of the cystic growth (Jaffe et al., 1942). The lesion may be primary with possible genetic predisposition (Leithner et al., 2004), or secondary to a pre-existing lesion such as fibrous dysplasia, hematoma from trauma, bleeding disorders, or within a pre-existing bone tumor (Leithner et al., 2004). Giant cell tumors are the most common cause in humans (Wu et al., 2011). In the case of the presenting filly, no bleeding disorder was identified pre-operatively and no bone tumor was observed on histopathological analysis. No previous trauma was reported although this could not be totally excluded as her early history was undocumented. The potential genetic predisposition of her family to bone lesions was not investigated. The etiology of this lesion remains unclear in this patient.

Absolute alcohol intracystic injection has been reported to be successful in the management of aneurysmal bone cysts in humans.
(Cottalorda et al., 2004). However, due to the multiloculated appearance of the cyst, the proximity of the tooth roots/buds and mandibular nerve, this non-invasive option was considered too risky. Autologous cancellous bone grafting was also considered a poor option due to the size of the cavity. The use of a synthetic bone graft thus provided an ideal solution. The technique employed in this case involved surgical curettage to remove the cyst followed by implantation of a tissue engineered scaffold to encourage repair of the mandible bone. This is the first time that this combination of techniques has been used to treat a large aneurysmal bone cyst. Due to the high risk of traumatic or pathologic mandibular fracture in this case, during surgery particular attention was paid to apply the scaffold to the area where the compact bone was extremely thin or perforated. Two additional sheets of the CHA bone graft substitute were also rolled and placed cranially and caudally to provide some healing in the cavity itself and underneath the bone flaps. The CHA scaffold displayed a perfect ability to adhere to the compact bone and demonstrated sufficient mechanical strength, flexibility and durability to withstand surgical handling and to be rolled and shaped to fit into the required spaces within the mandible cavity. The bone fenestration technique with the central bridge provided stability of this weakened mandibular bone during the curettage and early post-operative period. Although the blood supply to the bone flaps was completely
absent for the first days after surgery, no bone necrosis was noted and proper healing was evident on CT examination performed 3 months post-surgery. This maximum bone preservation approach likely contributed to the good recovery of this mare.

Bone repair was quantified at 3 months and 14 months post-surgery. The amount and quality of compact bone produced in 3 months was considered exceptional on comparative CT examinations. Analysis of the CT images revealed no trace of the CHA scaffold thus demonstrating the biodegradability of the scaffold and its successful resorption by the body as new repair tissue is formed. Prior to surgery, growth of the tumor within the mandible led to enlargement of mandible and thinning of the mandibular compact bone leaving the horse at significant risk of pathological fracture. Evaluation at 3 months confirmed that rapid repair of the mandible bone had occurred. Importantly, the compact bone was found to be continuous, with increased thickness compared to pre-surgery values and thus the risk of pathological fracture of the mandible was significantly reduced.

At 14 months, CT analysis revealed that further remodelling of the compact bone of the right mandible had occurred and a clearer definition between cortex and medulla was noted. This was confirmed
through mandible volume and area measurements, with the cavity volume being significantly reduced at 14 months compared to pre-surgery values. Importantly, no reoccurrence of the cyst was observed up to 14 months post-surgery. Oral examination revealed that on the right side, PM2 was significantly shorter than the left. It is likely the dental bud of PM2 was damaged during the curettage of the most rostral part of the cavity. Although curettage was carried out carefully with the use of a narrow suction tip to remove the fibrous tissue lining cystic material can be easily confounded with dental buds during surgery. While PM2 will require further monitoring it is unlikely to cause any significant issues.

These results demonstrate the benefits of the osteoinductive properties provided by the hydroxyapatite component of the scaffold combined with the biocompatibility and rapid degradation associated with the collagen component of the CHA scaffold. Notably, the bone remodelling observed here was achieved in the absence of additional osteogenic factors confirming the previously demonstrated osteoconductive and osteoinductive properties of this CHA scaffold and its ability to lead to tissue regeneration without the requirement for addition of growth factors such as BMP (Gleeson et al., 2010; Lyons et al., 2014; Murphy et al., 2014). This off-the-shelf, cell-free approach overcomes many of the limitations associated with currently
used autologous bone grafting procedures providing an ideal alternative from a clinical and regulatory stand-point.

5. Conclusion
This case study investigated the use of the CHA scaffold in the treatment of a multilobulated aneurysmal bone in a young horse and demonstrated its potential to enhance repair of the affected bone. Clinical follow-up at 3 and 14 months post-implantation revealed reduced enlargement of the operated mandible, initial thickening of the compact bone with no fluid filled area, and later remodelling of the compact bone with a clearer definition between cortex and medulla. Overall, the successful clinical outcome and enhanced bone formation observed in this craniomaxillofacial indication demonstrates the potential of this bone graft substitute for use in this and other equine indications and also for translation to human applications.

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