The CAM assay for human bone regeneration evaluation: The potential of Laponite Clay gel for growth factor delivery *ex vivo*

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Introduction

Bone fractures require the development of novel biomaterials which harness both skeletal regeneration and stable blood vessel supply[1] . In particular, injectable hydrogels have gained attention in tissue engineering for their similarities to natural extracellular matrix and their minimally invasive delivery. In the present study we have examined a novel, self-assembling, high-adsorptive clay hydrogel (*Laponite*) to deliver VEGF or BMP2 and trigger bone formation[2].

Following *in vitro* studies, it is essential to test biomaterials in the context of full animal physiology before the constructs reach the clinic. *In vivo* models are then required which typically involve invasive procedure in murine hosts. Thus, less sentient alternative *in vivo* models should be used when available[3].

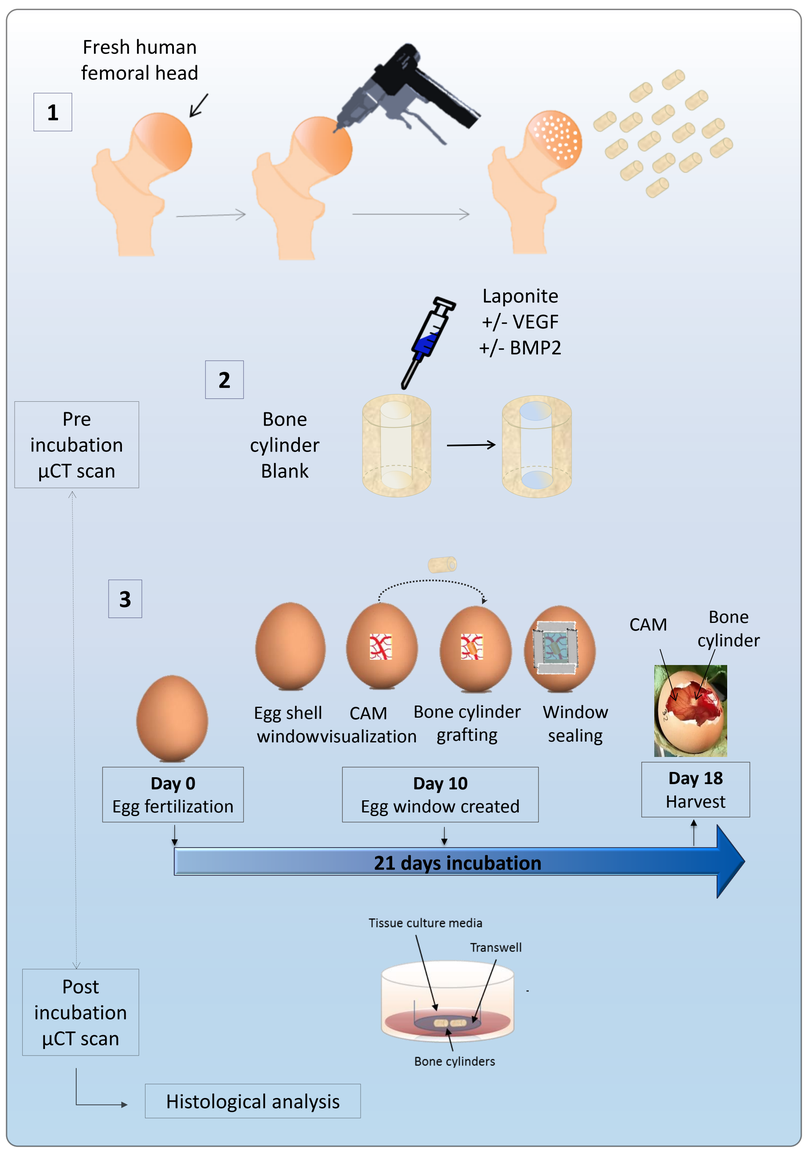
In this study, the **chorioallantoic membrane** of the chick embryo (**CAM assay**)[4] was used used to cultivate **freshly isolated human bone** thereby providing an alternative *in vivo* model using clinically relevant samples. Hence, our aims and objectives are:

1. To study the potential of the CAM to culture human bone tissue and providing a surrogate blood supply.

2. To develop a method to examine bone formation using the human-avian replacement model.

3. To evaluate the effect of Laponite as a hydrogel vehicle to deliver growth factors and enhance bone formation.

Methods



1) Human femoral heads were collected after surgery to freshly isolate bone cylinders. A 2mm drill defect was introduced on their core to mimic the injury area. All bone cylinders were µCT-scanned individually (18µm resolution).

2) The core of the bone cylinders was left empty (Blank) or perfused with Laponite, Laponite + VEGF or Laponite + BMP2.

3) Bone cylinders were implanted on the GFP-CAM for 7 days or cultured *in vitro* in basal media without serum.
Post-incubation µCT-scanning was performed and bone cylinders were processed for histology.

Results

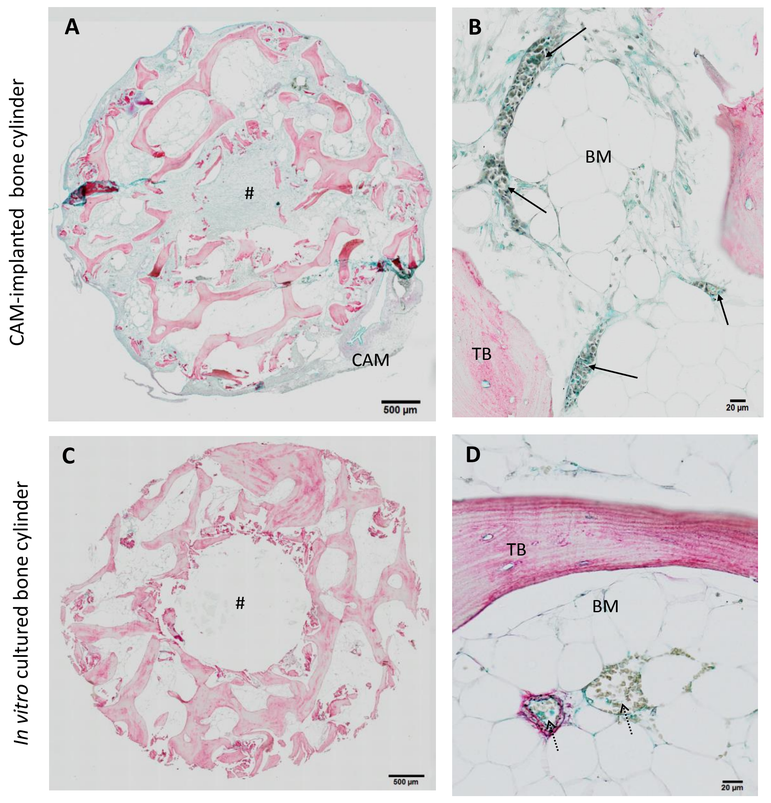


Figure 1: The CAM integrates with the human bone cylinder providing surrogate blood supply. Bone cylinders were cultured *in vitro* or CAM-implanted for 7 days. Paraffin sections were stained for Alcian Blue (proteoglycans) and Sirius Red (collagen). # indicates the defect region. Solid arrows indicate avian blood vessels carrying nucleated erythrocytes. Dashed arrows indicate enucleated erythrocytes of human blood vessels.

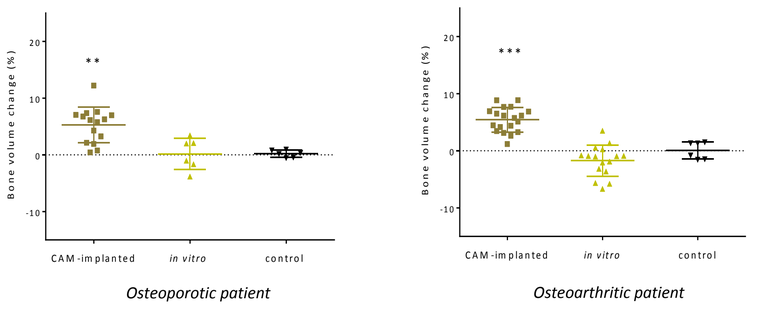


Figure 2: Bone volume significant increase of human bone cylinders following CAM-implantation. Bone cylinders were cultured *in vitro,* CAM-implanted for 7 days, or maintained at 4 ˚C as control. Each cylinder was µCT-scanned before and after under the same X-ray settings to quantify the relative bone volume change. Data representative of osteoporotic (n=3) and osteoarthritic (n=3) patients. Error bars indicate mean ±S D, $p<0.05$.

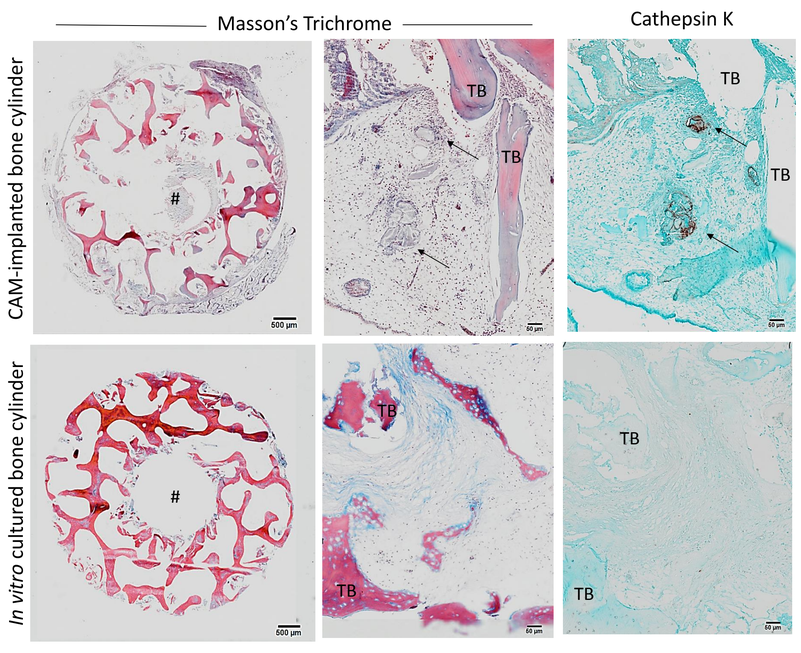


Figure 3: CAM implantation of bone cylinders results in osteoclast-mediated bone remodelling. Bone cylinders were cultured *in vitro* or CAM-implanted for 7 days. Consecutive paraffin sections were stained for modified Masson´s Trichrome (mineralised bone orange-pink, osteoid blue) and Cathepsin K immunostaining as osteoclast acitvity marker. # indicates the defect region. Solid arrows indicate Cathepsin K positive immunostaining.

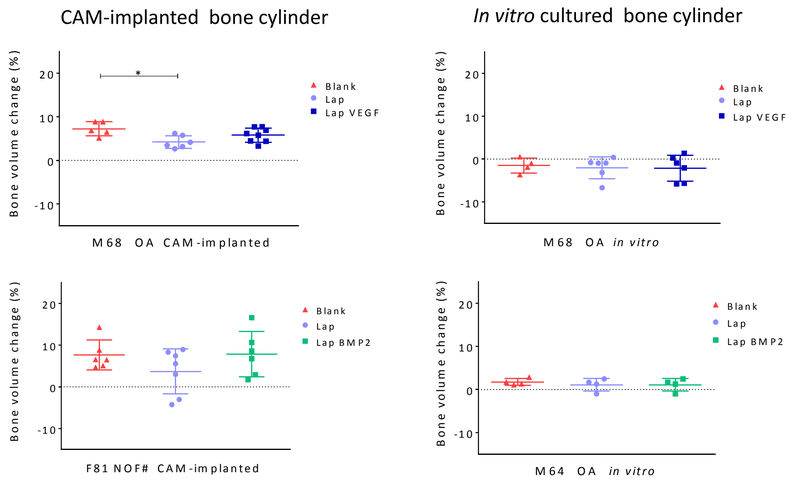


Figure 4: CAM-implanted bone cylinders allow detecting bone volume changes between treatments, but not *in vitro* culture. Bone cylinders were perfused with Laponite, Laponite +VEGF/BMP2 or Blank, and cultured *in vitro* or CAM-implanted for 7 days. Bone volume was quantified before and after 7 days incubation and the relative change was calculated for individual cylinders. Error bars indicate mean ± SD, $p<0.05$. One-way ANOVA with Tukey´s post hoc test.

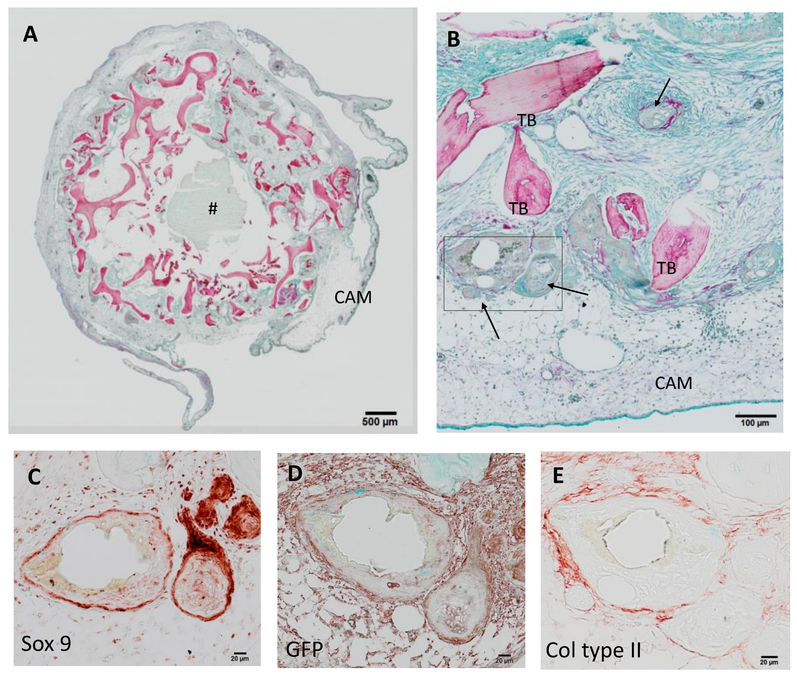


Figure 5: CAM-derived mesenchymal cell condensations and matrix deposition following BMP2 delivery on CAM-implanted bone cylinders. Bone cylinders were perfused with Laponite+BMP2 and CAM-implanted for 7 days. Consecutive paraffin sections were stained for modified Alcian Blue (proteoglycans) and Sirius Red (collagen) (A-B), Sox9 (C) GFP immunostaining to detect the cells from avian origin (D) and collagen type II (E). # indicates the defect region. Solid arrows indicate mesenchymal cell condensations between human and avian tissue. .

Conclusions

The current study has demonstrated the validity of the CAM as an ex vivo model to culture human bone and study bone regeneration. The combination of this *ex vivo* model with the use of high resolution computed tomography and histological analysis provides a powerful method to study the effect of novel constructs in bone tissue engineering.

In summary, the main conclusions of this study are:

1. The CAM is able to perfuse the human bone tissue with rapidly growing blood vessels and hence a context for bone remodelling.

2. Implantation of the human bone cylinders on the CAM results in significant bone formation

3. Laponite modulates the deposition of mineralized tissue in the bone cylinders.

4. The addition of BMP2 resulted in the formation of mesenchymal cell condensations (Sox9+) from avian origin and extracellular matrix deposition.

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