Establishing a 3D *Ex vivo* Culture System for Investigations of Bone Metabolism and Biomaterial Interactions

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Summary
A 3D load-providing culture system is presented, which enables human cancellous bone explants to be studied ex vivo. Bone integrity and activity was demonstrated during 2 to 3 weeks of culture. Viable osteocytes and bone specific markers could be identified. Culture conditions were improved using a serum free medium containing TGF-β3. Therefore this system gives the opportunity to study bone biology, hormonal effects as well as biomaterial interactions ex vivo prior to animal studies.

Keywords: human, bone, osteocytes, osteoblasts, loading culture chamber, reduction, replacement, toxicity testing, biomaterials, implants, validation

Background Information

Importance of 3D *ex vivo* bone studies using a mechanical load providing bioreactor

It is well known that strain is one of the main factors that can influence bone architecture and mass. The correct dosage of an applied mechanical load can therefore act as an anabolic stimulus on bone. On the other hand disuse of bone (astronauts at zero gravity; bed rest patients; immobilisation) can cause a severe loss of mass and strength in normal load bearing bones. It is hypothesised that the cells which can sense and transduce the information produced due to mechanical loading are the osteocytes. These cells are connected to others via their fluid-filled lacuna-canalicular system and are proposed to communicate with each other as well as with adjacent osteoblasts, bone lining cells and osteoclasts. Commonly, 2D cell cultures are used to investigate bone related questions. These systems lack mechanical load and the complex interplay between the different cell types. Therefore, there is a need for a 3D bone culture system as the naturally occurring bone cell interaction, in combination with the presence of the different cell types, can not be achieved in 2D. With the use of a Zetos bioreactor (created by DB Jones, Marburg, Germany and EL Smith, Madison, Wisconsin, USA) load, that mimics the naturally occurring pattern, can be applied to ex vivo cultured cancellous bone explants, which can be supplied with their individual culture medium. This opens new possibilities in bone research. Beside the use in pretests of biomaterial integration, this system will also be a potential means to study basic bone biology, bone diseases and effects of different drugs on e.g. osteoporosis. It has been demonstrated that this model can be used to maintain viable human osteoporotic bone ex vivo. Current methods of studying osteoporosis which use sheep models aim to simulate osteoporotic bone using steroids, special diets and sometimes removal of the ovaries which are usually very distressing for the animal. This culture system enables more rigorous in vitro testing, reducing the number of animals needed within such a study.

The system – model systems, material processing and usage

Cancellous bone due to its porosity is the bone of choice for a 3D culture system. Diffusion of fluids and nutrient supply can be achieved easier than using 3D cortical bone. Cancellous bone is present in sufficient mass at different origins. Three model systems are used within our working group – distal end of ovine femurs, bovine distal metacarpals and human femoral heads (approved by Ethic Commission Graubünden 18/02). No animals were sacrificed specifically for this work, bovine material was collected from the local slaughterhouse and ovine material was used from animals after sacrifice for other experiments. The processing of each bone includes cutting 7 mm thick slices with the use of an Exakt 300 band saw, then cores of 9.5 mm diameter are bored from the sections with a Synthes drill bit. Finally these are cut parallel to 5 mm height with a Leica annular saw (Davies et al., 2006). After removing debris with repeating washing steps, each core is placed in its individual culture chamber and perfused with its individual culture medium with a flow rate of 0.1 ml/min. Mechanical loading can be applied with the Zetos bioreactor (Jones et al., 2003) (Fig. 1) by inserting a chamber into the load-

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ing device. The loading procedure used is performed daily for a duration of 5 min using a complete jump wave form (300 cycles, 1 Hz, 4000 µstrain). After long term culture cores can be harvested and analysed in order to answer the specific question.

Bone characterisation and protein synthesis on cultured bone explants

After culture, cores can be either fixed in 70% ethanol prior to dehydration and Technovit 9100 embedding (Yang et al., 2003) for histochemical analysis; or fixed and decalcified prior to cryosectioning, to perform immunohistochemical evaluation on 6-12 µm sections. Histological sections (6 µm thick) of bovine, ovine and human material after culture are comparable to fresh bone concerning matrix and cell integrity, as demonstrated by a variety of stains. The presence of noncollagenous proteins, such as osteopontin, osteonectin and osteocalcin could be localised immunohistochemically.

Protein synthesis could be detected using [3H]-glycine incorporation combined with SDS-page and Western blot analysis as well as autoradiography of embedded sections, demonstrating cell viability and activity during 14 days of culture within the system. In addition, the presence of the unstable propeptide of collagen-I could be detected on 12 µm cryosections of 14 days cultured human bone cores (Fig. 2).

Influence of loading and long term culture on bone viability

Investigating bone viability of 3D bone explants after culture can be difficult. The bone matrix autofluorescence interferes with many fluorescence labelling techniques. Radioactive labelling of protein synthesis has inherent risks and can take many weeks to develop images. Both of these methods require embedding or decalcification procedures prior to cutting. We have focused on using a Lactate dehydrogenase assay (LDH) to detect an essential cytoplasmatic enzyme. The high stability of LDH allows cutting of fresh cores prior to staining. After fixation, visualisation of purple stained viable cells can be performed. We could quantify osteocyte viability per area of bone matrix with an optimised method using the natural autofluorescence of the bone matrix to enhance the staining contrast (Stoddart et al., 2006) (Fig. 3).

Using the LDH viability method on human cancellous bone cores, it could be demonstrated, that a daily applied load could maintain higher osteocyte viability after 7 and 14 days of culture

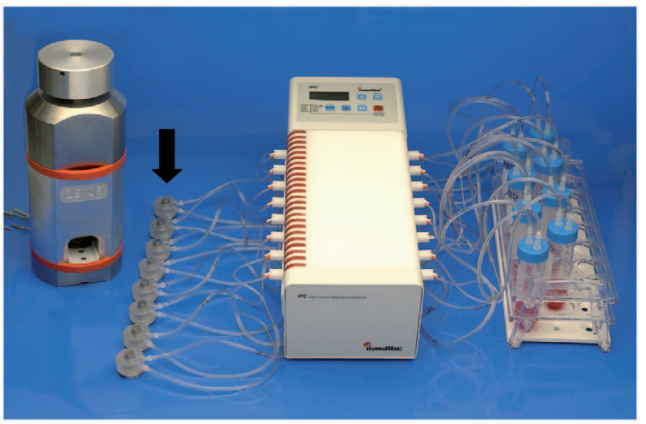

Fig. 1: Zetos culture system which comprises of a loading device (left), and a set of diffusion culture chambers (arrow), each with their own media supply fed from a reservoir (right) through Tygon tubing with the aid of a peristaltic pump (white box).

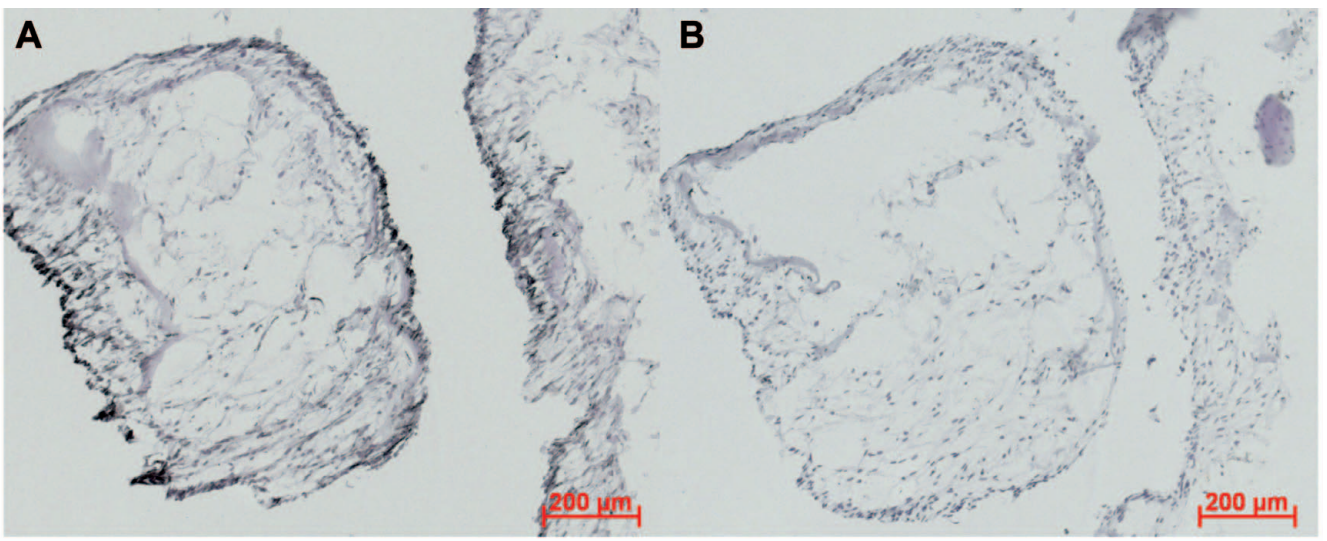

Fig. 2: Immunohistochemistry of procollagen-I on cryosections from 14 days cultured human bone explant (64 years, male) within the Zetos system.
A) Procollagen-I staining, B) Negative control without primary antibody

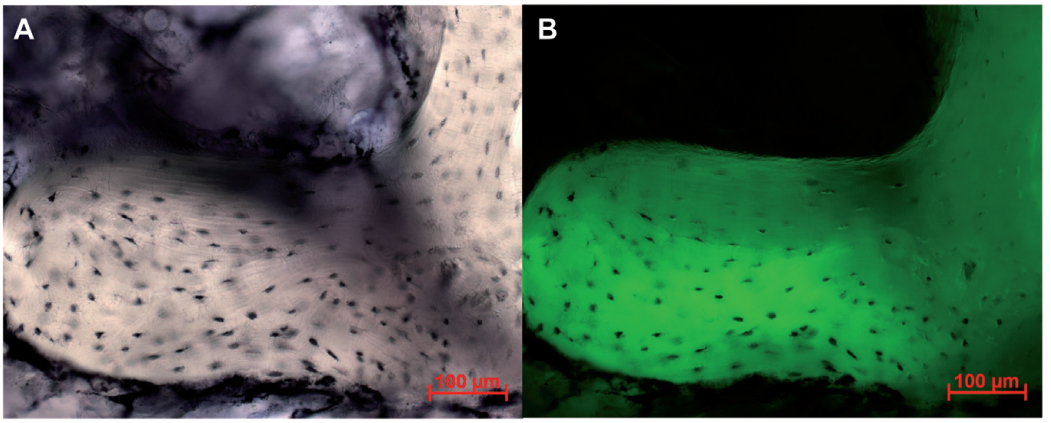

Fig. 3: Two representative pictures from LDH stained middle sections of one human (64 years, male) loading experiment visualised with Axioplan microscope.
A) Brightfield image, B) Fluorescence image (515 – 565 nm emission filter)

in comparison to unloaded control cores. Therefore the bioreactor itself improves 3D culture conditions.

Effect of TGF-β3 on osteocyte viability and the use of a new defined culture medium

The TGF-β superfamily consists of cytokines that can affect different cellular events, such as cell growth, differentiation, apoptosis etc. In bone TGF-β was shown to increase collagen-I synthesis as well as matrix apposition and therefore enhance the total bone mass. We chose to look at the effects of TGF-β3, as it was demonstrated by ten Dijke et al. to be more potent, when comparing general DNA and collagen synthesis to the more commonly studied TGF-β1 (ten Dijke et al., 1990). During the culture of human bone cores with a DMEM based culture medium containing 10% fetal calf serum (FCS) and 15 ng/ml TGF-β3, higher osteocyte viability maintenance could be achieved which exceeded the effect caused by daily loading alone. This led us to investigate the effect of this factor within a medium without serum, which is normally thought to cause a higher cell death rate due to reduced factor concentrations found within serum containing medium. Serum free medium including 15 ng/ml TGF-β3 was supplemented with insulin, transferrin, selenium and a defined lipid source to provide a defined environment of factors essential for cell survival and activity. Analysis of the centre of cores cultured for 14 days either within serum containing or serum free medium, revealed a similar level of osteocyte viability (Fig. 4). A common phenomenon during culture of 3D bone explants is the formation of an inconsistent fibrous tissue on some / all surfaces of the cores. The fibroblast-like cells covering the core will decrease nutrient supply of the underlying cells as well as secrete factors that could influence the culture conditions. Using a serum free medium the chance of surface fibrous tissue formation was reduced in comparison to serum containing medium. Therefore the culture within a serum free medium achieves a more defined culture environment and maintains the level of osteocyte viability achieved with the use of FCS within culture medium. Additionally, this medium was also proven to show procollagen-I synthesis during 14 days of culture as seen with the serum containing control, demonstrating cell activity.

Improved media distribution with the use of a new chamber design

Further improvements of culture conditions were focused more technically on modifying the existing culture chambers. As sticking of bone cores to baseplate or piston surfaces of the chambers can occur, this may cause declined media distribution on the core surfaces. Therefore baseplate and piston surfaces where modified by applying 0.2 mm deep channels in a negative honeycomb pattern. Perfusion experiments using a disulfide blue containing medium revealed a more even media distribution using these “honeycomb surfaced” chambers. Viability investigations using these chambers with the use of the defined serum free medium are currently under process.

Conclusion and future possibilities

The *ex vivo* loading Zetos culture system was validated to culture cancellous bone with osteocytes, osteoblasts, osteoclasts and bone marrow cells in their natural 3D relationship to each other. This system also has advantages in reducing the variability, cost and ethics behind *in vivo* studies. Most experiments evaluating biomaterial integration with bone and soft tissue are conducted within animals. Therefore implants for human use can now be tested *in vitro* with applied load (and this is underway). Only the most successful biomaterial/implant would continue for animal trials, reducing the number of animals required for such studies. The development of a realistic 3D loaded system, whereby the candidates could be eliminated before *in vivo*

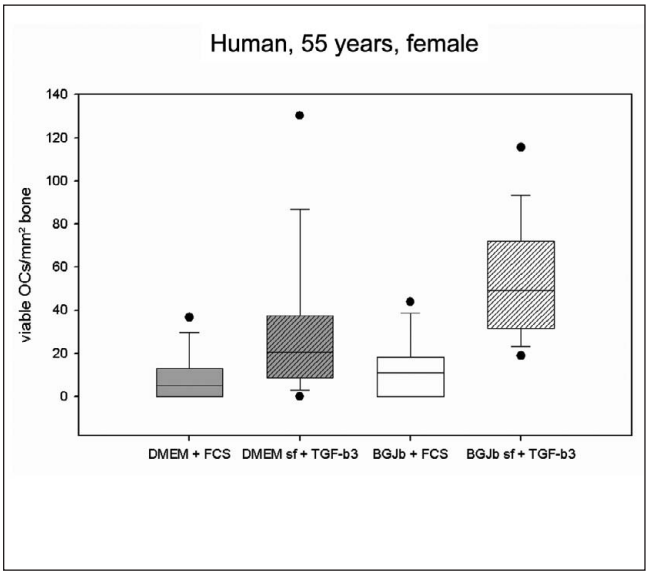

Fig. 4: Graph of one human loading experiment (55 years, female) investigating the effect of serum free medium on osteocyte viability.
The number of viable osteocytes per mm2 bone matrix area at the centre of the cultured bone explants was compared between DMEM + 10 % FCS (grey bar) *versus* DMEM serum free + 15 ng/ml TGF-β3 (striped grey bar); and between BGJb + 10 % FCS (white bar) *versus* BGJb serum free + 15 ng/ml TGF-β3 (striped white bar).

studies would greatly reduce the number of animals. Therefore the system has potential study bone-biomaterial interactions, as well as bone biology in normal and osteoporotic human or animal bone, bone biomechanics, and the effects of drugs, hormones or growth factors on cancellous tissue, making it an essential laboratory aid for the future.

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