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Original Article

Hylonome

Zetos: A culture loading system for trabecular bone. Investigation of different loading signal intensities on bovine bone cylinders

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Abstract

The objective of this investigation was to test the effects of different intensities (1000, 1500, 2000, 3000, and 4000 μstrain) of a physiological loading signal (jumping) on trabecular bone stiffness and osteoid thickness using the ZETOS culture and loading system. Fourty eight bovine bone samples were randomised equally across 6 groups: 5 loading groups and 1 control group. The bone samples were cultured for 26 days (DMEM high glucose medium) and subjected to mechanical stress on 23 days. The stiffness of the samples was determined each day before loading in the loading groups and every 3rd day in the control group. The stiffness measurements in the loaded groups were significantly higher than in the control group. The degree of stiffness increased continuously throughout the observation period in the 1500, 2000, and 3000 μstrain groups. Maximum stiffness was achieved in the 4000 μstrain after a very short time (8th loading day) and then remained constant to the end of the investigation. The osteoid thickness in this group was, however, not higher than in the 2000 and 3000 μstrain groups. The 2000 μstrain group showed the highest proportion of newly formed osteoid. The amounts of osteoid deposited in the 2000, 3000 and 4000 μstrain groups were significantly greater than in the control group. Moreover, a correlation between increasing intensity of the signal and increase in osteoid deposition was observed. Histological investigations were conducted on non-decalcified bone and showed a well-preserved trabecular architecture and cell morphology.

Keywords: ZETOS, Trabecular Bone, Stiffness, Osteoid, Bioreactor

Introduction

The positive influence of mechanical loading on the bone metabolism with improved bone healing or remodelling can be clearly demonstrated in the veterinary and clinical setting. However the processes involved in mechanical signalling remain in the most part obscure.

The evidence from animal models suggests that mechanical loading leads to enhanced bone strength and that there is a direct positive correlation between functional loading and the extent of bone regeneration, even with only short periods of loading[1].

A few researchers have measured bone response in animals exposed to precisely quantified loads at distinct skeletal sites. Rubin and Lanyon applied dynamic loading to pins inserted in the ulnae of turkeys (isolated wing preparation model) with the same load being applied to each turkey ulnae. Bone hypertrophy was directly proportional to the applied load[2]. Turner et al. used a four-point bending apparatus to apply bending forces to the lower right tibia of 9-month-old female rats. He observed what appeared to be a loading threshold for the activation of osteoblasts at about 1050 μstrain. There was a linear increase in formation on the endocortical surface after the strain threshold was surpassed[3]. Both of these *in vivo* models demonstrated a threshold response and a dose-response relationship between load and bone formation. All of these effects *in vivo* are observable after a period of several weeks to months however the

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techniques used are possibly not sensitive enough to show small effects below the threshold of detection. It thus remains unclear how early changes (within minutes), assumed to be part of the mechanotransduction pathway, can be related to histological changes many weeks later.

Bone tissue contains a large number of different cell types which interact to maintain the bone metabolism[4]. These include monocyte-derived osteoclasts and osteoblast bone stem cells which during the remodelling process arrive with the blood vessels, the blood vessel cells themselves and nerve cells. Many of the interactions of all the cell types have not been fully investigated as many experiments are performed in cell-culture models[5-8].

The ZETOS System® [9] was developed against this background. In a specially designed culture chamber with a dynamic bioreactor (3-D culture), the system allows physiological and mechanical signals to be applied throughout the experiment lasting up to 28 days[10]. This explant culture model also enables the testing of the controlled application of osteogenic factors, such as PTH, TGFβ, Vitamin D_3, or ß-glycerophosphate and retinoic acid.

Previous studies have shown some limitations of the system. Davies et al. investigated viability[10]. Briefly the outside 0.5 to 1 mm dies quickly due to the effect of cutting. The middle also dies off quickly due to lack of oxygen nutrients and waste exchange, leaving an envelope between which appears to be healthy. Indeed David et al.[11] have shown that in the system the adipose/osteoblast switch through PPARγ is activated in a similar manner to excercised mice. However, depending on the medium used, at about 14-28 days stem cells from the healthy living layer start to populate the surface and cover the explant with a fibrous layer. This uses up the available oxygen and cuts off diffusion. After this the inside quickly becomes necrotic.

Using the ZETOS System®, it is presently possible to perform dynamic stimulation in a frequency range of 0.1 to 50HZ with a compression precision of ±0.5 to ±1.5 µstrain. A piezoelectric actuator (PZA) is used for dynamic stimulation, with which it is also possible to perform real-time measurements of force and compression. A quasi-static loading regimen can also be applied via the PZA, enabling the stiffness of the bone samples to be determined.

The aim of the present study was to determine whether trabecular bone samples responded with physiological reaction patterns to force (data taken from a loading platform simulating jumping). The physiological reaction patterns were reflected by the increase in apparent stiffness of the bone cylinders, expressed here in terms of the relative stiffness showing the increase in bone mass in the form of newly-formed osteoid and therefore demonstrating appositional bone growth.

Materials and methods

The Zetos® culture and loading system for trabecular bone

This *ex vivo* system, developed by Professor DB Jones (Marburg, Germany) and Professor E Smith (Madison, Wisconsin, USA), was designed to culture and load trabecular bone explants in their near natural environment to measure the extent to which it is affected by mechanical and chemical stimuli.

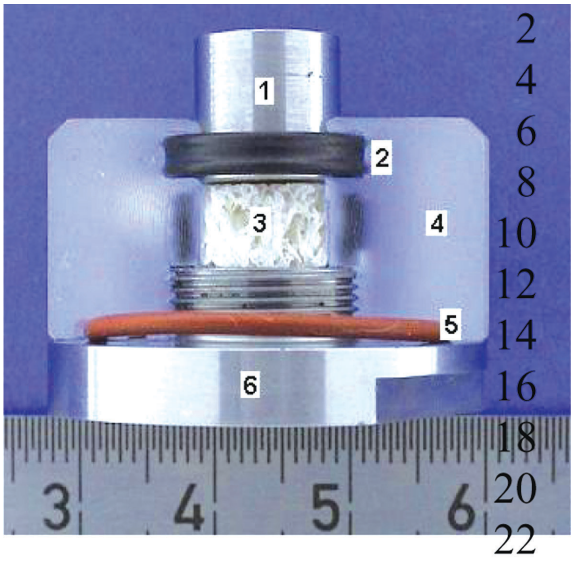

Figure 1. Cross-section of loading chamber with bone sample. Experimental apparatus in hot room at 37°C.

This investigation used cylinders of trabecular bovine ulna (obtained at slaughter) as organ cultures with a diameter of 10 mm and a depth of 5 mm. We used the bovine model for ease of access, genetic similarity (from one local herd) and not diseased, which is not the case for human bone. Such cylinders are a 3-D *ex vivo* system which bridges the gap between *in vitro* and *in vivo* methods[10] and may contain up to 2 million cells. Other groups are working with human-, ovine- or bovine sternum explants[10,12,13]. The system enables the behaviour of and interactions between different cells (such as precursor cells, osteoblasts, osteoclasts, osteocytes, and granulocytes) to be studied outside the living animal when different stimuli are applied.

The system also enables the application of different, defined load signals, such as the walking signal, the jumping signal or rectangular signals, at any frequency or number of cycles. It also incorporates real-time recording of compression and the forces used to achieve this with a precision of ±50 nm.

Different properties of bone cylinders can be determined using this system, such as the actual bone stiffness, viscoelastic properties, and the fracture strength.

The ZETOS System can be configured with a frequency range from 0.1-50 Hz and a maximum load up to 4500 N. Forces of 4500 N, however, are very much higher than the fracture strength of the bovine bone samples used in this investigation. The system was therefore calibrated with a maximum load of 1500 N with a compression precision of ±50 nm. A typical maximal force to achieve 4,000 µstrains was in the region of 450 N, large variations (±40%) being observed within the same bone.

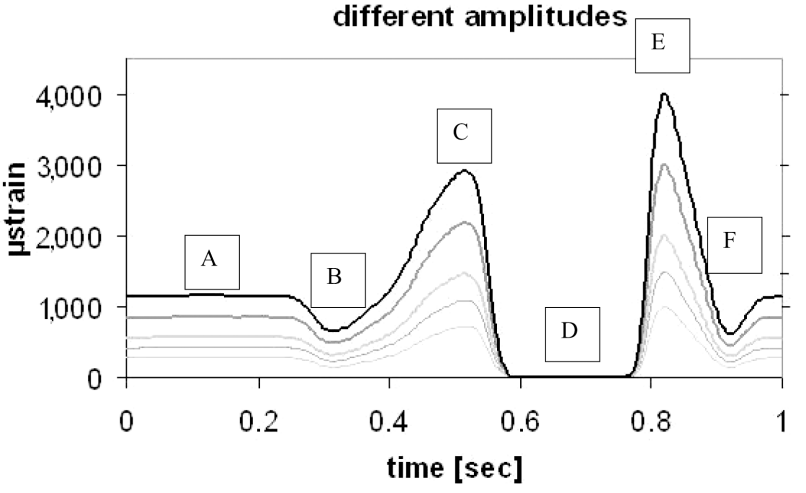

Figure 2. The different phases of the jumping signal. The phases can be described as follows. A: Foot on the ground; B: Gaining impetus; C: Jumping up; D: Free in the air; E: Landing; F: Cushioning.

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Control	1,000 µstrain	1,500 µstrain	2,000 µstrain	3,000 µstrain	4,000 µstrain

Table 1. Groups and loading intensity.

In the present investigation, a loading signal corresponding to that of jumping with a frequency of 1Hz and load duration of 5 minutes was applied for 300 cycles daily was applied. The signal was tested in different groups with intensities ranging from 1,000-4,000 µstrain.

Preparation of the bone cylinders

The bone material used for this investigation was taken from the ulna of a 20-month-old cow and was culled immediately after slaughter in a slaughterhouse. After direct transport to the laboratory, slices of trabecular bone 7-8 mm thick were sawn from below the epiphyses using a diamond bandsaw (Exact, Norderstedt) under sterile conditions. Using a specially designed diamond-coated hollow drill, cylinders with a diameter of 10 mm were drilled out of the bone slices. The bone cylinders were then cut down to a thickness of 5 mm using a plane-parallel saw. During the entire sawing and drilling procedure, the saw band, drill and bone were continuously cooled with sterile 0.9% sodium chloride solution and rinsed to remove bone dust and prevent the bone samples from drying out. The bone cylinders were then washed in an agitating rotation washer for 2 x 10 minutes with Earle's Salt Solution and then 1 x 20minutes with Earle's Salt Solution containing 500,000 IU/L penicillin, 375,000 IU/L streptomycin and 4 mg/L amphotericin B at 37°C.

The bone cylinders were then placed in the sterile bone chambers and connected to the medium reservoir with a Tygon pipe system. The culture medium was DMEM high glucose with 10% foetal calf serum, 2mM glutamine, 50,000 IU/375,00 IU/L penicillin/streptomycin, 5 mM ß-glycerophosphate, 5 mg/L vitamin C phosphate, 10 mM Hepes, and 0.12 g/L sodium hydrogen carbonate with an adjusted pH of 7.25. The culture medium was supplied with an Ismatec 24-channel peristaltic pump at a rate of 7 mL per hour (Figure 1).

Conduct of the experiment

The 48 bone cylinders were randomly assigned to 6 groups of 8 samples (Table 1).

Group 1 served as a control group with any loading. Only the apparent stiffness was determined every 72 hours.

The assigned load intensity was applied for 5 minutes daily at a frequency of 1 Hz to the bone samples in Groups 2-6. The apparent stiffness was determined before the load was applied. With the jumping signal as the loading regimen, loading started 48 hours after preparation.

The flow rate of the culture medium can be freely adjusted using the 24-channel peristaltic pump. We chose a flow rate of 7 mL/h for this investigation because previous studies had shown that this rate does not stimulate the bone samples

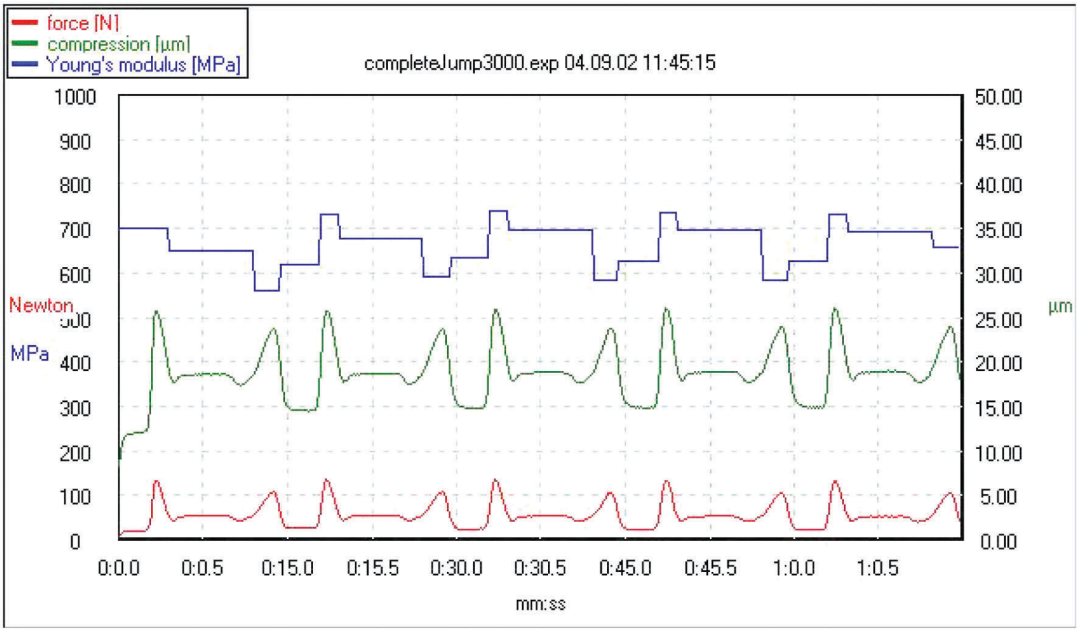

Figure 3. Loading protocol for a jumping sequence at an intensity of 3,000 µstrain.

via shearing forces. The further procedure was the same in all groups, including the control group. The culture medium was changed and the pH determined every 72 hours.

On day 12, the bone samples were labelled with 30 µg/mL calcein and on day 21 with 45 µg alizarin S to enable appositional bone growth to be determined on the degree of fluorescence of the stain deposited in the trabeculae.

Used medium was stored at -80˚C for the later determination of alkaline phosphatase and LDH as markers of apoptosis.

Load signals

The load signal (jumping signal) can be described graphically as follows (Figure 2).

The entire loading procedure is controlled by a Windows®-compatible program specially developed for this device. The observation period of 25 days comprised 23 days on which the loading was applied in all groups. The bone samples were subjected to the jumping sequence at the assigned intensity for 23 successive days for 5 minutes. (Figure 3).

The loading protocol recorded the loading sequence and the resulting compression together with the force required to achieve the compression in Newton and the apparent stiffness (Young's module/E-module) in mega-Pascal.

Apparent stiffness of the bone samples/E-module

The apparent stiffness of each bone sample was determined immediately before the dynamic stress. The value obtained is converted into a practical statistic independent of geometry that reflects a pure material quality that can easily be compared with other common materials. This statistic is called the E-module or Young's module.

At the beginning of this quasi-static measurement, the PZA is first brought into contact with the loading piston in the chamber via a threaded screw.

Applying voltage to the PZT actuator results in an expansion which, depending on the blocking force can be in the from 0.001Hz to the kilohertz range for the type used in the Zetos. The maximum compression during the quasi-static measurement is 20 µm. The tension does not then abruptly drop, but declines over 10 seconds describing a cosinus curve to avoid dynamic stimulation with higher frequencies. During this period, data on the force and compression are continuously recorded. The apparent stiffness is then printed out as a regression curve, whereby the same partial interval (in the range of 15-20 µm) is always used for each bone sample from all data available. The following figure shows the results of such a quasi-static measurement, with the mean and standard deviation. The force interval in which the curve follows a linear course is shown (designated here by [fa, fb]). The stiffness is represented by the reciprocal gradient of the regression curve shown here in bold (Figure 4).

Following the quasi-static measurement, the monitor displays a measurement protocol similar to the following figure. For each bone sample in the control group, 8 such measurement protocols including the apparent stiffness were produced, and 23 were produced for each loading group (Figure 5).

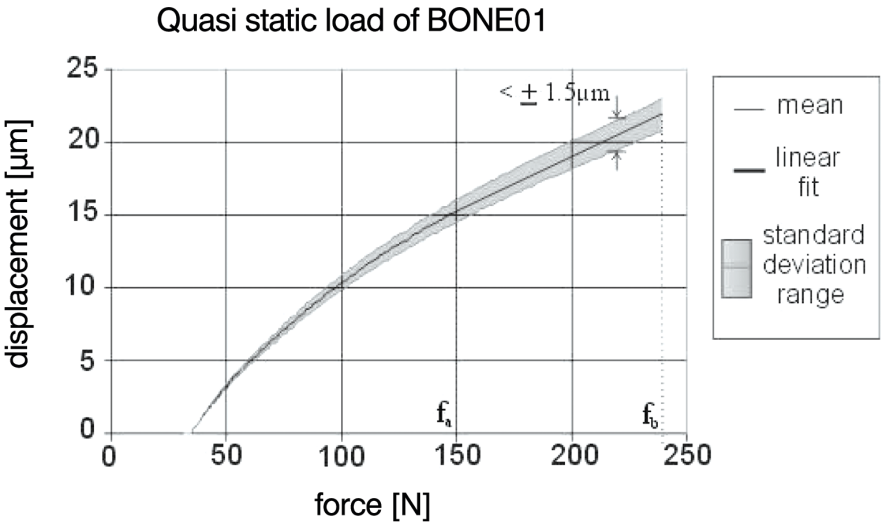

Figure 4. Example of the graphic display of a quasi-static measurement to determine the apparent stiffness.

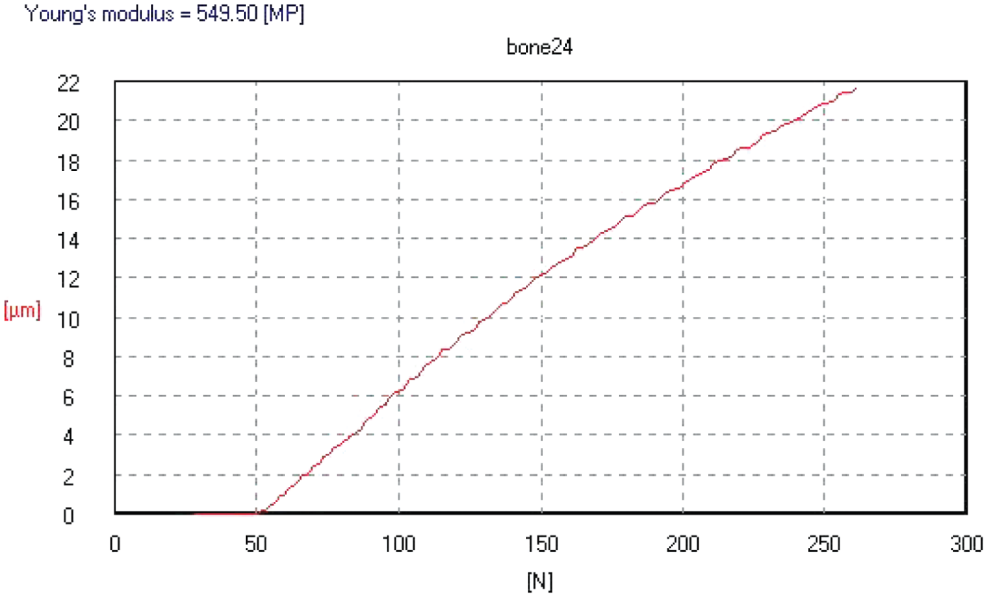

Figure 5. Typical measurement protocol of a bone sample for the determination of apparent stiffness.

Fixation of the bone cylinders

At the end of the investigation, the bone cylinders were fixed in 1.4% paraformaldehyde solution for 24 hours at 4°C. The bone samples were then washed in 0.04 M phosphate buffer +10% sucrose solution twice for 8 hours at 4°C. The samples were dehydrogenated using an increasing alcohol dilution series for 24 hours per alcohol step and were then degreased in two steps with xylol.

The bone cylinders were embedded using several steps. After finishing the increasing alcohol series and leaving the bone cylinders in xylol as an intermediate medium, they were embedded in Technovit® 9100 New (Kulzer) according to the manufacturer's specifications. The polymerization phase was performed in a freezer at -8 to -15°C for 12-48 hours, depending on the degree of hardness reached. Microscopic sections were obtained with the "Polycut E" solid microtome produced by Reichert-Jung®. Following deplastification, the samples were stained with trichrome dye according to Goldner, and sealed.

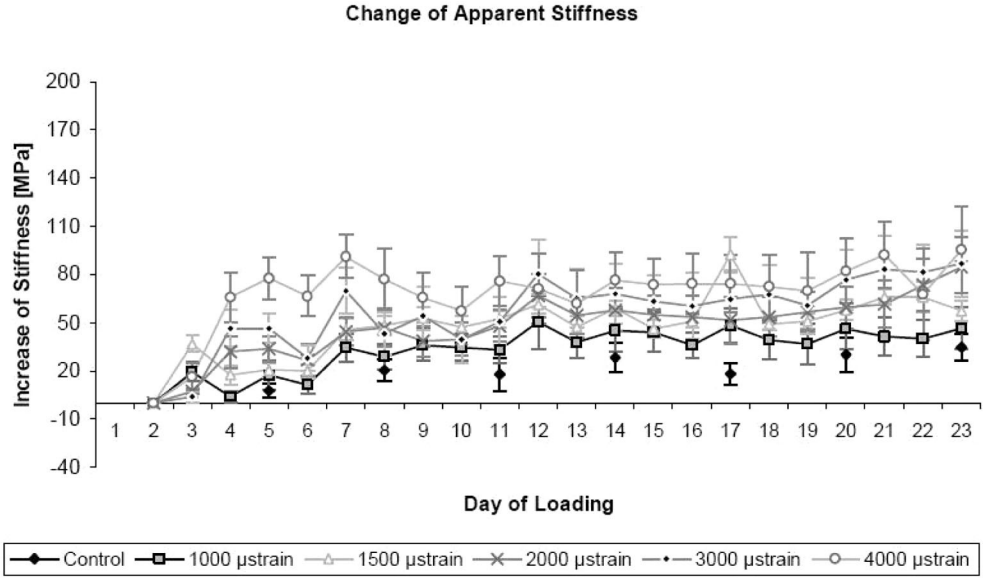

Figure 6. Arithmetic mean (n=8) for 22 measuring days for the loading groups and 8 days for the control group. Changes in apparent stiffness and standard deviation.

Trichrome staining according to Goldner

Staining according to Goldner is one of a number of trichrome staining methods and is a standard method used in bone morphometrics. One of its particular qualities is that it makes it easy to distinguish between mineralised and demineralised bone matrices. It also stains cells very well. Mineralised tissue and collagens are stained bright green, osteoid red, cell nuclei blue-black, and cytoplasm reddish brown.

To test whether any changes in stiffness found were due to biological responses or simply due to physical effects (e.g., compacting of the tissue due to loading) a series of experiments were conducted with dead tissue (killed by heating or 0.9% KCl).

Statistics

The results presented here were statistically evaluated by the Institute of Medical Biometrics and Statistics of the Philipps University in Marburg, Germany using SAS Release 8.0.

All results were presented graphically as mean and standard deviations. A general mixed linear model for repeated models with unbalanced data was used to display the analysis of effects of loading over time.

Load and time were included as fixed variables. Since this model requires normally distributed data, they were first logarithmically transformed, which brought them close to normal distribution. A global test on differences between groups and time points, if appropriate, was first conducted. In each case, the F statistic and the p-value were calculated. A p value of <0.05 indicated generally significant differences between groups.

Individual groups were compared using linear contrasts according to Tukey. Because there were so many groups, the data had to be alpha-adjusted for multiple testing, with the result that the differences between some groups did not reach statistical significance.

Single factor analysis of variance with six load levels was used for the osteoid evaluation. The results were also logarithmically transformed before testing to ensure normal distribution. The level of significance for these tests was $p=0.05$.

Results

Apparent stiffness of bone samples (E-module)

The apparent stiffness or the E-module was determined on each loading day before dynamic stimulation. The results from the first loading day were ignored, which left 22 measurements for each sample for the 23-day observation period. The stiffness of the control samples was determined every 3 days (72 hours) and there were therefore 8 measurements available for this group.

The following figures show the change in the mean apparent stiffness in each group over the observation period (Figure 6).

The apparent stiffness increased in all loading groups over the observation period. The apparent stiffness in the control group also increased. However, in comparison to the loaded groups, after an initial increase, the level remained fairly constant. No decrease in stiffness occurred. Over a relatively short period, the group with 4,000 µstrain showed the maximum increase in stiffness and then remained on a plateau. In contrast, the groups

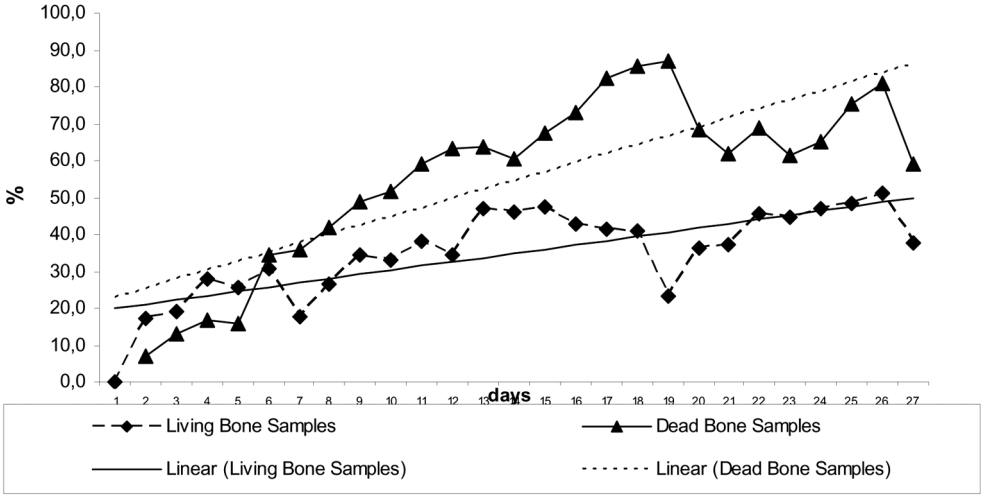

Figure 7. Increase in stiffness in dead bone samples compared to living bone samples with loading of 3,000 μstrain (jumping signal).

with the loading of 2,000 and 3,000 μstrain showed continuous increases in stiffness over the entire observation period, although the increase was obviously more marked up to the halfway point (day 11 or 12) than in the second half.

Experiments with dead tissue

When the bone cores were first killed using either heat or O.9% KCl, and loaded as for the viable groups, a significant *increase* change in relative stiffness over the vital tissue was observed (Figure 7).

The test for differences between the 6 groups showed a significant global group effect with an F statistic of 18.04 (p=0.0001), and a significant global time effect with an F-statistic of 68.61 (p=0.0001). The following results emerged for the comparison of the groups using linear contrasts (Table 2).

All loading groups except Group 1 differed significantly from the control group. Amongst the loading groups, only Groups 1 and 5 differed significantly from one another.

The increase in stiffness in the first half of the investigation differed significantly between the group with 4,000 μstrain and the control group (p=0.0198). There were no differences between the individual groups for the increase in stiffness in the second half of the investigation.

The osteoid

The osteoid bands were measured manually using the Image®ProPlus software. To enable differentiation between mineralized bone substance and osteoid, the histological preparations of all loading and control samples were stained with trichrome dye according to Goldner (see Material and methods). Osteoid is stained red and mineralized bone substance green. Using Image®ProPlus, 8 fields of each sample were evaluated at a magnification of 100x. Arithmetic means were then calculated for each sample and group from the measurements collected. The groups are compared in the following figure (Figure 8).

Comparison of groups	p-value	Significant
0 and 1	>0.05	no
0 and 2	0.0002	yes
0 and 3	0.0128	yes
0 and 4	0.008	yes
0 and 5 and 6	0.0005	yes
0 and 6	0.0098	yes
Between other groups	>0.05	no

Table 2. Test for differences between the 6 groups.

The highest degrees of osteoid deposition were seen in the group with maximum load of 2,000 μstrain, followed by the groups with 3,000 and 4,000 μstrain, and the lowest degree in the control group. A significant global group effect with an F-statistic of 5.83 (p=0.0004) was observed.

The subsequent individual comparisons using linear contrasts according to Tukey showed significant differences between the control group and the loading groups with 2,000, 3,000 and 4,000 strain.

Correlation between apparent stiffness and osteoid formation

The increase in apparent stiffness during the investigation and the thickness of osteoid formation were compared using the arithmetic means in each group to establish the extent of any correlation between the two. The figure below shows the correlation (Figure 9).

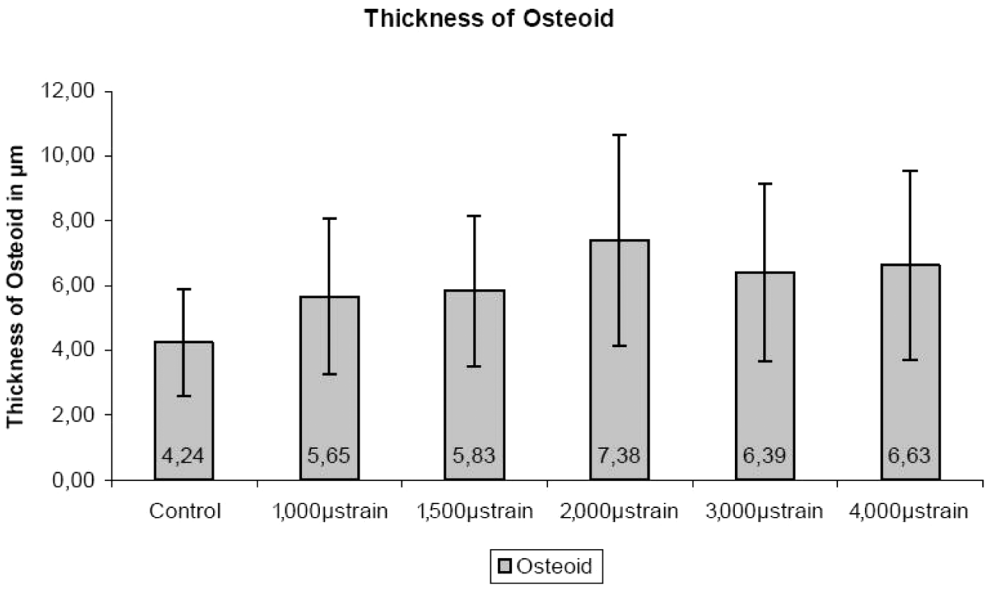

Figure 8. Arithmetic mean for osteoid deposition with standard deviation.

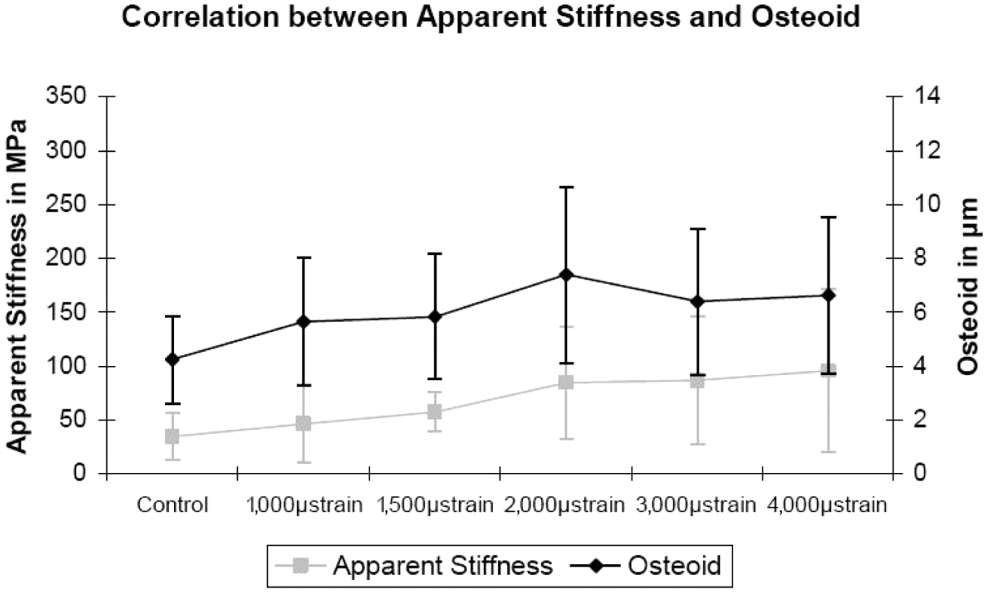

Figure 9. Correlation between apparent stiffness and osteoid thickness (arithmetic mean and standard deviation).

The change in apparent stiffness is dependent on the load. The degree of osteoid formation also appears to correlate with the intensity of the load. It can therefore be assumed that the apparent stiffness of the bone samples increases with an increasing load due to the formation of osteoid. An exception to this was the group with a maximum load of 2,000 µstrain, where greater amounts of osteoid deposition were observed than in the 3,000 and 4,000 µstrain groups. There was, however, a greater degree of scatter in this group.

Discussion

Bovine trabecular bone cylinders were subject to cyclical-mechanical stress corresponding to jumping at different load intensities for 4 weeks to determine the increase in stiffness and appositional bone growth.

The roughness of the bone surface and plane parallelity were determined in advance to ensure even application of the loading signals. The sawing and drilling techniques used

resulted in plane parallelity with a variance of up to 6 µm on a cylinder surface with a diameter of 10 mm. The roughness of the trabeculae was determined using a confocal microscope (Leica) and the mean was between 8-16 µm on the bony part of the cylinders. The sapphitre pistons used to contact the bone and apply the forces were sealed using x rings which allowed several degrees of freedom of movement and allowed compensation for the non parallelity.

Mechanical stimulation may result in an increased flow in the fluid in the bone (this has never been measured). Either the hypothesised flow or the mechanical loading results in an increased survival of the osteocytes in the Zetos chambers[13,14]. The culture medium contained 5 mM ß-glycerophosphate as an osteogenetic and mineralisation factor and 10% purified foetal calf serum. Despite loading signals of different intensities, this concentration was not sufficient to produce evident deposition of two fluorochromes applied (calcein, alizarin S) in the bone cylinder. Hence it is not clear from where the increase in stiffness arises. Dead, loaded tissue shows a significant increase in stiffness over the loaded groups. It is known that mineralisation in bone is an ectopic process which is regulated by inhibitors, e.g., pyrophosphates which are in turn degraded by alkaline phosphatase. Nevertheless the differences between the two groups suggest that some vital biological process is still occurring in the tissue. However we do now know how much mineralization is necessary to produce the observed, small, changes. Stiffness of a cylinder scales with the radius squared, so small, unobservable changes might still be responsible for our observed results (Figure 10).

The increase in osteoid was determined, and here we found a significant difference from the control group.

The values obtained for the increase in osteoid using histological sections showed that mechanical loading resulted in an increase in bone deposition. Mature osteoblasts develop into flattened bone-lining cells on the mineralised bone surface. These protect the mineralised bone matrix from extracellular fluid and are directly in contact with the bone channels and therefore with the osteocytes. Osteocytes and bone-lining cells are connected metabolically and electrically by gap-junction proteins, termed connexins[15] (Figure 11).

The direct communication between active osteoblasts or bone-lining cells with the osteocytes in the bony matrix is assumed to be principle that factors induced by mechanical forces applied to bone are passed on to the osteoblasts or bone lining cells by stress signals produced by the osteocytes. These take up the greatest proportion of the systemic and local signals and react with the corresponding response.

If bone is to respond to physiological stimuli as a functioning unit, vital cells must remain present in the organ culture. The marked increase in osteoids in the groups with loading shows, on the one hand, that adequate physiological stress was applied and, on the other, that the cell complex in parts of our organ culture was adequately supplied with nutrients. Other studies with the Zetos[11,12], have shown that similar responses of the bone to loading occur as with animal

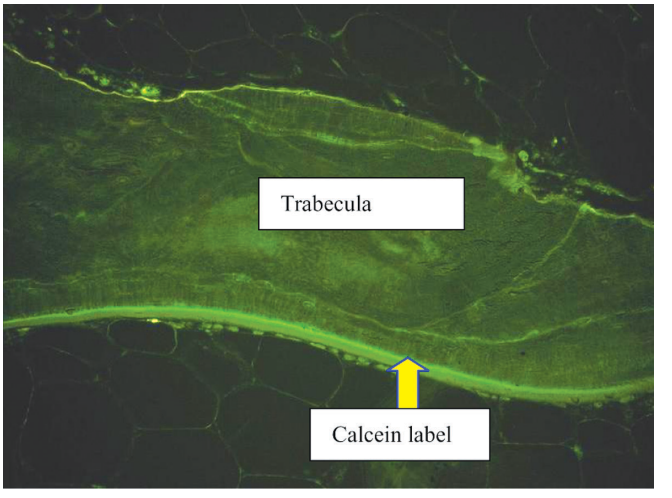

Figure 10. Calcein-alizarin S labelling of trabecular bone. The calcein labelling was performed on day 12 of the investigation and the alizarin S labelling on day 21.

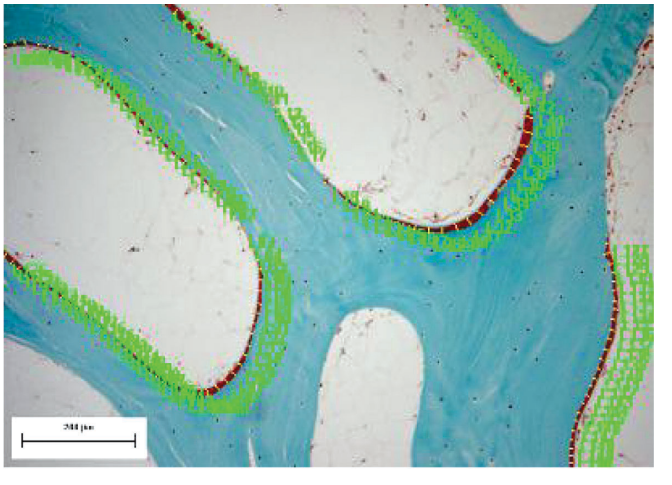

Figure 11. Example of Masson-Goldner staining with evaluation of osteoid thickness.

experiments, indicating that the vital parts of the tissue can still demonstrate physiological responses. Murray et al.[16] have extracted viable megakaryocyte stem cells from the Zetos bone cylinders within 14 days of culture. Studies are underway to determine how best to maintain viability using different media, different cutting techniques and different geometries (e.g., larger surface area to volume ratios) to achieve the best overall viability of the stem cells in the bone and in the blood vessels. We have not investigated the viability of the nerve cells which we assume also play a highly important role in bone cell biology, but suppose that these die during the machining process.

Mechanical stress appears to play a decisive role in the formation of new bone. A recent publication by Mann et al.[13] reported an increase in the rate of apoptosis of osteocytes in the absence of stress. Another report, also using the Zetos, found that this protection due to loading was enhanced with TGFβ[14]. These results are in line with human and animal studies[17]. The clinical consequences would be reduced bone mass and density, and resulting reduction in the weight-bearing capacity of bone.

The principal aim of the present investigation was not to demonstrate that our 3-D culture method preserved the vitality of the osteocytes and of the cell complex. By detecting glycogen (PAS-Alcian blue staining) on the histological section in some cases, we did, however, demonstrate the presence of vital osteocytes and osteoblasts.

The increased osteoblast activity with preserved vitality leads us to presume that the trabecular bone samples were supplied with adequate amounts of nutrients. This is supported by findings published by Dodd et al.[18], who observed reduction in the number of viable osteocytes as a result of the absence of mechanical stress *in vivo*. This was, however, reversible after applying stress.

In our investigation, the osteoblast function measured on the increase in osteoid thickness in trabecular bone was significantly increased in relation to the loading intensity.

The greatest degree of osteoid deposition was observed in the group with the maximum load of 2,000 μstrain, followed by the groups with 3,000 and 4,000 μstrain. The lowest degree of osteoid deposition was seen in the control group, as was to be expected. We found an increase in bone stiffness in dead tissue, higher than in partially vital tissue. Mineralisation in bone has to be prevented actively rather than induced actively, and when it occurs it is directed to specific sites. The medium contained beta glycerophosphate and we would expect ectopic calcification to occur. This osteoid formation is more of an indicator of vital bone formation than stiffness *per se* if no acurate assessement of cell vitality is made.

The increase in stiffness, like the osteoid thickness, was dependent on the loading intensity. It can therefore be assumed that the apparent stiffness of the bone samples increases with an increasing load intensity, and that this is determined by the amount of osteoid formed. We were therefore able to demonstrate a positive correlation between osteoid thickness and increase in stiffness of trabecular bone.

The outcome of our investigation, therefore, has real relevance to the clinical situation: adequate stress on a segment of the skeleton, regardless of whether in the post-operative situation or as a preventative measure, can lead to improved bone architecture and can also accelerate bone healing.

One problem in our investigation was that the expected deposition of two fluorochromes applied (calcein and alizarin S) did not occur in the trabecular part of the bone cylinders. This was presumably because there was too little ß-glycerophosphate in the medium. Future investigations will include different media compositions to enable better evaluation of the osteoblast activity.

Histological investigations of the upper and lower sides of the bone cylinders after stress at the end of the investigation showed that the surfaces had been smoothed by the daily loading. At present, it is also not possible to assess to what extent this may have affected the vitality of the bone samples.

References

1. Lanyon LE. Using functional loading to influence bone mass and architecture: objectives, mechanisms, and relationship with estrogen of the mechanically adaptive process in bone. Bone 1996;18(Suppl.1):37S-43S.
2. Rubin CT, Lanyon LE. Regulation of bone mass by mechanical strain magnitude. Calcif Tissue Int 1985; 37:411-7.
3. Turner CH. Mechanical loading thresholds for lamellar and woven bone formation. J Bone Miner Res 1994;9:87-97.
4. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349-55.
5. Wolf K, Quimby MC, Pyle EA, Dexter RP. Preparation of monolayer cell cultures from tissues of some lower vertebrates. Science 1960;132:1890-1.
6. Yeh CK, Rodan GA. Tensile forces enhance prostaglandin E synthesis in osteoblastic cells grown on collagen ribbons. Calcif Tissue Int 1984;36(Suppl.1):S67-71.
7. Jones DB, Leivseth G, Tenbosh J. Mechano reception in osteoblast-like cells. Biochem Cell Biol 1995;73:525-34.
8. Zaman G, Suswillo RF, Cheng MZ, Tavares IA, Lanyon LE. Early responses to dynamic strain change and prostaglandins in bone-derived cells in culture. J Bone Miner Res 1997;12:769-77.
9. Jones DB, Broeckmann E, Pohl T, Smith EL. Development of a mechanical testing and loading system for trabecular bone studies for long-term culture. Eur Cell Mater 2003;5:48-59; discussion 59-60.
10. Davies CM, Jones DB, Stoddart MJ, Koller K, Smith E, Archer CW, Richards RG. Mechanically loaded *ex vivo* bone culture system "Zetos": systems and culture preparation. Eur Cell Mater 2006;11:57-75.
11. David V, Martin A, Lafage-Proust MH, Malaval L, Peyroche S, Jones DB, Vico L, Guignandon A. Mechanical loading down-regulates peroxisome proliferator-activated receptor gamma in bone marrow stromal cells and favors osteoblastogenesis at the expense of adipogenesis. Endocrinology 2007;148:2553-62.
12. David V, Guignandon A, Martin A, Malaval L, Lafage-Proust MH, Rattner A, Mann V, Noble B, Jones DB, Vico L. Tissue Eng Part A 2008;14:117-26.
13. Mann V, Huber C, Kogianni G, Jones D, Noble B. The influence of mechanical stimulation on osteocyte apoptosis and bone viability in human trabecular bone. J Musculoskelet Neuronal Interact 2006;6:408-17.
14. Simpson AE, Stoddart MJ, Davies CM, Jähn K, Furlong PI, Gasser JA, Jones DB, Noble BS, Richards RG. TGFbeta3 and loading increases osteocyte survival

in human cancellous bone cultured *ex vivo*. Cell Biochem Funct 2009;27:23-9.
15. Doty SB. Morphological evidence of gap junctions between bone cells. Calcif Tissue Int 1981;33:509-12.
16. Clarke MC, Savill J, Jones DB, Noble BS, Brown SB. Compartmentalized megakaryocyte death generates functional platelets committed to caspase-independent death. J Cell Biol 2003;160:577-87.
17. Aguirre JI, Plotkin LI, Stewart SA, Weinstein RS, Parfitt AM, Manolagas SC, Bellido T. Osteocyte apoptosis is induced by weightlessness in mice and precedes osteoclast recruitment and bone loss. J Bone Miner Res 2006;21:605-15.
18. Dodd JS, Raleigh JA, Gross TS. Osteocyte hypoxia: a novel mechanotransduction pathway. Am J Physiol 1999;277(3Pt.1):C598-602.