European Journal of Morphology, October/December 2005; 42(4/5): 208–216

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ORIGINAL ARTICLE

Tracking the changes in unloaded bone: Morphology and gene expression

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Abstract

Bone formation and growth are controlled by genetic, hormonal and biomechanical factors. In this study, an established rat disuse osteoporosis model, hindlimb-suspension (HLS), was used to relate morphological change and gene expression to altered mechanical load in the underloaded femora and the ostensibly normally loaded humeri of the suspended rats (39 days old at onset; 1, 3, 7 and 14 days suspension). Morphological change was measured by labelling new bone formation with fluorescent agents during the experimental period and subsequent histological analysis of bone sections post-sacrifice. Hindlimb suspension reduced both the total amount of bone present, assessed as cross-sectional area, and the bone formation rate at the mid-diaphysis of the unloaded femora while no significant effect was found in the loaded humeri. In addition, the femora of the suspended animals were found to have a markedly increased circularity as a result of unloading. A sensitive semi-quantitative method of gene expression analysis, involving the creation of SMART^{TM} cDNA arrays, was successfully implemented. This technique amplified all populations of mRNA to levels where they could be assessed using standard molecular biology protocols. Gene expression patterns of two candidate genes, c-fos and osteocalcin were assessed in periosteal tissue. Altered gene expression patterns were identified and tracked over the suspension period. The altered levels of both candidate genes were found to be consistent with the changes observed in the histological analysis.

Keywords: *Morphological change, hindlimb suspension, mechanobiology, gene expression*

Introduction

Bone is self-repairing and can alter its properties in response to the stresses placed upon it. The process by which bone adapts to mechanical loading is generally known as functional adaptation. Mechanically, adaptation works to continually tailor bone to the loads placed upon it, an example of which would be the increased bone mass in tennis players' dominant arms (Jones et al. 1977). Young soccer players have also been found to have a higher bone mass compared with non-players, the soccer players had significantly higher BMD of the total body (2.7%), lumbar spine (6.1%), the dominant and nondominant hip. The largest differences were found in the greater trochanter on both sides (dominant, 16.5%, nondominant, 14.8%) (Soderman et al. 2000).

Conversely, reduced loading results in a reduction in the bone mass present. Clinically, this can cause problems when the mechanical force necessary to maintain bone mass is removed. This commonly occurs in illness (bed rest, paralysis) or when bone around prosthetic implants is stress shielded. In these cases, the loads applied to the bones are significantly reduced and resorption occurs to adapt the bone to this new loading regime. In extreme cases this can lead to the prosthesis loosening and the requirement for revision surgery (Jacob & Huggler 1980). Long term immobilisation or repeated periods of short term immobilisation can have serious detrimental effects on the skeleton (Mosekilde et al. 2000). Measurements of the bone mineral density (BMD) in the spine, femur neck, trochanter and pelvis of subjects in these bed rest studies have shown a reduction of between 0.9 and 1.3% per month (LeBlanc et al. 1990).

Despite much research in the area, the mechanobiology of bone is not very well understood.

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ISSN 0924-3860 print/ISSN 1744-4241 online © 2005 Taylor & Francis
DOI: 10.1080/09243860600737261

Decreased loading has been studied using a number of methods, including immobilisation, spaceflight and unloading. Lanyon (1982) observed significant decreases in the mechanical properties (bone mass, width and curvature) in the right hindlimb of rats which had undergone denervation compared to both the contra-lateral limb and those of controls. Uhthoff & Jaworski (1978) studied the response of bone to non-traumatic immobilisation, by encasing the right forelimb of young dogs in plaster. After 32 weeks, bone mass stabilised after an initial reduction of 30–40%. In a later study on mature dogs, similar disuse caused an approximately 33% reduction in bone mass. Underloading has been shown to cause a reduction in cross-sectional area (Uhthoff & Jaworski, 1978; Spengler et al. 1983) but has little effect on bone length (van der Meulen et al. 1995). Site and compartment-specific changes in bone have also been found in mature adult rats subjected to hind-limb suspension (Bloomfield et al. 2002).

This study investigated the effects of altering the mechanical load applied to the long bones of growing rats. Skeletal unloading of the hindlimbs was achieved using a rat hindlimb suspension model (HLS) first designed in NASA by Morey (1979) and subsequently used by a number of investigators (Globus et al. 1984; Bikle et al. 1994a; van der Meulen et al. 1995). This model unloaded the hindlimbs while maintaining weight-bearing forces on the forelimbs. A number of studies have attempted to utilise HLS to examine the effect of unloading on mRNA levels. Cells have been shown to have a memory of their previous mechanical loading history. Bone marrow stromal cells (BMSCs) extracted and cultured from the marrow cavities of bones which had been unloaded via HLS had a reduced proliferation and bone forming rate (Kostenuik et al. 1997; Machwate et al. 1993). In addition, Kostenuik et al. (1999) demonstrated that BMSCs from rats that were hindlimb suspended for 5 days had 50% less c-fos and 35% less osteocalcin mRNA, after 20 days culture, than BMSCs from control rats. This mirrored a reduction in the mineralisation of the cultured cells from suspended rats. This result contrasts with that of Machwate et al. (1993) who reported that after 14 days unloading the osteoblast phenotype was unchanged. However this could be explained since studies have shown that bone formation has returned towards normal following 12–14 days continued unloading (Globus et al. 1986). However care must be taken when interpreting these results as they are not a direct measurement of gene function. The effects on gene expression of mechanical reloading of hind limbs after 14 days of tail suspension was also examined by Matsumoto (1998). Reloading of the hind limbs caused a transient increase within 2 h of the expression of c-fos in periosteal cells. It was suggested that mechanical unloading causes an impairment of periosteal bone formation by impairing the expression of c-fos in periosteal cells. Tanaka et al. (2004) analyzed the effect of unloading by HLS on the anabolic action of parathyroid hormone (PTH) in the tibia of growing mice and found that unloading promoted osteoclastogenesis and seemed to delay the progression of osteogenic differentiation with a reduced increase of c-fos mRNA in bone marrow cells. Iqbal & Zaidi (2005) examined the molecular regulation of mechanotransduction and demonstrated that mechanical stimulation affects the physiological activity of osteoblasts and leads to increased c-fos and osteocalcin production. Kesavan et al. (2005) evaluated the skeletal response to mechanical loading of two inbred mouse strains (C57B1/6J (B6) and C3H/HeJ (C3H)) and found that at 4 and 8 days of loading, expression of a number of bone formation genes including osteocalcin was increased and correlated these changes with subsequent increases in bone mineral density.

In this study, hind limb suspension was used to relate morphological change and gene expression to altered mechanical load in the underloaded femora and in the loaded humeri of the suspended rats. The working hypothesis in this study was that unloading would result in a reduction in the expression of two genes associated with bone formation, osteocalcin and c-fos.

Materials and methods

Animals

Following 5 days of acclimatization, under Irish government licence (Ref. B100/2195), groups of 39-day-old Sprague–Dawley rats were randomly assigned to basal control (B), age matched control (C) or suspended (S) groups. Group B ($n = 6$) were sacrificed at the onset by over-anaesthetisation with CO_2 followed by cervical dislocation. Since all the animals were young growing rats, this basal control group revealed whether a measured change in a particular parameter in groups C and S was a change due to hindlimb suspension or simply a change due to growth of the young rats. As a result differences in body weight between unloaded and age matched control groups were minimal but were significant in comparison to Group B.

Conditions of altered load

Group S were hindlimb suspended to elevate and unload the hindlimbs while maintaining load on the forelimbs. Traction tape was attached and secured along the base of the tail. This tape was then

connected to a series of overhead pulleys allowing the animal's pelvis and hindlimbs to be elevated. This system of pulleys allowed the animal to use its front limbs to move freely in any direction around the cage and was vital for maintaining health. In addition, as the animals grew, the sides of the cages were raised to ensure the hind limbs remained unloaded. Groups of animals were hindlimb suspended for periods of up to 2 weeks (1, 3, 7 and 14 days, $n = 6$ in each group) concurrently with age-matched pair-fed controls ($n = 6$ in each group). Animals from both groups were sacrificed at each of these time points by over-anaesthetisation with CO_2 followed by cervical dislocation. After sacrifice, both loaded humeri and underloaded femora were disarticulated and cleaned of excess tissue. The periosteum was then dissected away from the mid-diaphysis of the right femur using a sterile scalpel and placed in sterile cryo-vials (Nalgene cryogenic vials, Nalgene Company, USA) filled with RNALater (Ambion Inc., TX, USA), and stored at -70°C. This process protected the samples' mRNA from degradation. Total time from sacrifice to snap-freezing of all periosteal samples was less than 10 min. The whole bone samples were then placed in separate vials and frozen at -20°C.

Morphological change

To quantify the degree of periosteal modelling, remodelling and bone formation, intraperitoneal injections of 30 mg/kg of oxytetracycline were given a day before the experimental period began and 30 mg/kg of calcein blue (Rahn 1977) given the day prior to sacrifice. Fluorochromes bind to exposed calcium and as they label the periosteal and endosteal surfaces at the time of administration, they can be used to calculate bone formation rates by relating the amount of new bone formed as a percentage of the total bone area.

Morphological changes due to altered load were assessed by histological analysis. The frozen right bones were measured using a vernier calliper on a bed of ice and their midpoints determined using standard anatomical points. In the humeri the length measured was from the distal surface of the trochlea to the proximal surface of the humeral head, while in the femora from the distal surface of the condyles to the superior surface of the greater trochanter. The bones were then sectioned transversely into two halves across the mid-diaphysis using an electric circular saw (Miniplex Triplex, Miniplex, France) transferred to two new cryo-vials and re-stored at -70°C for later analysis. The distal halves of both right long bones were stored for gene expression analysis while the proximal halves of the right femora and humeri were embedded in methyl-methacrylate, sectioned transversely using a diamond saw to create 50-μm thick sections and mounted on glass slides. Five sections per bone were analysed using an integrated morphological measurement package (Lucia-Image^{TM}). Under normal incident light, mean cross-sectional area (CSA) and circularity ($4\pi \times$ Area/[Perimeter]^2) was measured in all groups for each bone cross-section. CSA was calculated by subtracting the endosteal area (marrow cavity area) from the periosteal area. Circularity was calculated using the expression above with perimeter calculated from a measurement of the periosteal circumference. Using UV epifluorescence microscopy, the degree of periosteal and endosteal modelling was measured to calculate the Bone Formation Index (BFI). The BFI was defined as the amount of new bone formation divided by the original bone area i.e. periosteal BFI = new periosteal bone area/total bone area. The BFI was calculated individually for the periosteal and endosteal surfaces and the sum of the two used to calculate a total BFI. This parameter is a method of examining the effect of unloading on the growth of long bones that isolates the formation response from that of resorption. Unfortunately, using this method it is not possible to calculate a resorption index, as it is not possible to measure how much bone has been resorbed since day 0. However, when the results from the bone formation index are examined in conjunction with the bone cross-sectional area results, it is possible to infer the resorption response to altered mechanical loading.

Gene expression analysis

Periosteal tissues were homogenised and total RNA was extracted using the Ultraspec II RNA isolation kit (Biotecx Labs Inc., USA). RNA quality was then assessed visually by electrophoresis on denaturing agarose gels. Patterns of gene expression (osteocalcin and c-fos) were assessed using the SMART^{TM} protocol (Figure 1). SMART^{TM} is a commercially available method of analysing gene expression (Clontech, USA) and uses the general properties of mRNA in a modified Polymerase Chain Reaction to amplify all mRNAs present in a sample up to a predetermined cycle point (Chenchik et al. 1996; Zhumbayeva et al. 2001). This amplified SMART^{TM} cDNA is then concentrated by ammonium acetate/ethanol precipitation and transferred onto triplicate Hybaid^{TM} nylon membrane in a Northern 'dot' blot. A further check was also carried out at this point by creating a genomic ladder of sequentially diluted volumes (with each sequential volume being half the previous one) of cDNA ranging from around 2 μg to 36 ng. This ladder served to test whether observed changes in hybridisation signal were proportional to changes in gene expression and also to detect the sensitivity of the method. The triplicate SMART^{TM}

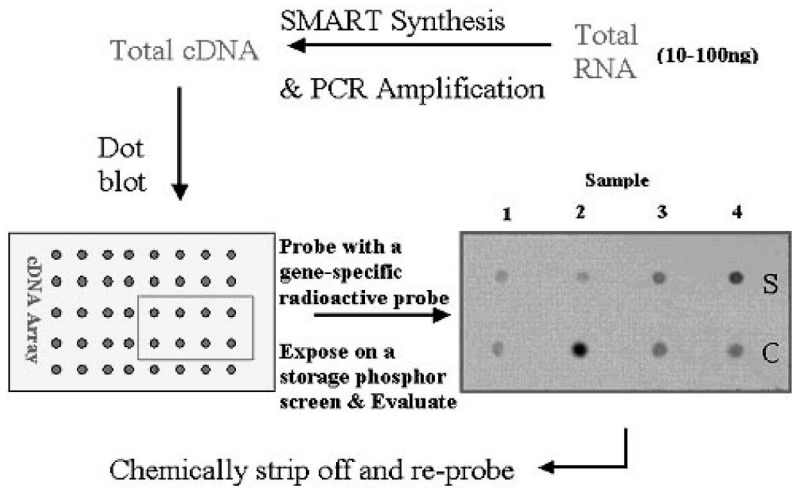

Figure 1. Modified SMART^{TM} protocol for gene expression analysis.

cDNA membranes were then sequentially hybridised with gene-specific radioactive probes created by standard PCR amplification from cDNA extracted from rat periosteal tissue. Levels of expression of each gene were assessed by exposing a phosphorus storage screen to the membrane and measuring the signal intensity on a phosphor-imager (Typhoon 8600 Imaging Workstation, Amersham Biosciences, UK). By comparing the intensities of these signals relative to those of two constantly expressed housekeeping genes (*GAPDH* and *RNRPS9*), information was obtained showing whether the expression of a gene has been up- or down-regulated as a result of the altered loading conditions (i.e. in a semi-quantitative manner). For each gene the normalised data from all three triplicate arrays are pooled. The data for each candidate gene was analysed for each of the two housekeeping genes separately. The two sets of data (relative to the two housekeeping genes) are then pooled and analysed collectively. This acts as a further check to prevent spurious false positives, as a significant alteration in gene expression should be present in all three comparisons (relative to the two individual housekeeping genes and in combination).

Statistical analysis

All data measured were statistically analysed using the statistics software Intercooled STATA (StataCorp. 2001). Linear regression analysis and ANOVA (Analysis of Variance) were performed on all data sets. The median values were used in the statistical analysis as it a more robust indicator of the central tendencies of the data than mean values. All graphical data are displayed in the form of box plots. Box plots display the median, inter quartile range and any outliers. The line in the middle of the box represents the median or 50th percentile of the data. The box extends from the 25th percentile, $x_{[25]}$, to the 75th percentile, $x_{[75]}$, the so-called interquartile range, IQR. The lines or 'whiskers' emerging from the box extend to the upper and lower adjacent values. The upper adjacent value corresponds to the largest data point less than or equal to $x_{[75]} + 1.5$*IQR. Conversely the lower adjacent value is defined as the smallest data point greater or equal to $x_{[25]} - 1.5$*IQR. Any values more extreme than the adjacent values are plotted individually. Linear regression analysis and ANOVA (analysis of variance) were performed on all data sets. The choice of which statistical method to use was governed by the following premise. In cases where it is assumed that there is a linear effect on a particular data set (e.g. for an increase in the independent variable, such as time, there is a linear response in the dependent variable) both ANOVA and linear regression analysis were used. In these cases, ANOVA and linear regression analysis will yield similar results. However, if a pulsatile effect was thought likely (e.g. in cases where for an increase in the independent variable, such as time, there is a transient response in the dependent variable) only ANOVA was used to analyse the data.

Results

Morphological change

All animals increased their body weight with increasing age over the entire experimental period. However, there was no statistically significant effect of HLS on the body weight of the two groups at any point (ANOVA, $P = 0.1944$; Linear Regression Analysis, $P = 0.594$). Morphologically, HLS resulted in a limited change in the mid-diaphyseal growth patterns in the humeri but significant change in the mid-diaphyseal growth patterns in the unloaded femora. Table I shows the morphological data for the humerii in both the control and suspended

Table I. Morphological data for the humerii in both the control and suspended groups.

		3 Days		7 Days		14 Days	
	0 Days	Control	Suspended	Control	Suspended	Control	Suspended
Cross-sectional area (mm^2)	2.48 ± 0.15	2.39 ± 0.15	2.22 ± 0.08	2.43 ± 0.31	2.75 ± 0.23	2.68 ± 0.14	2.69 ± 0.27
Circularity $4\pi \times$ Area/[Perimeter]^2	0.87 ± 0.03	0.88 ± 0.02	0.91 ± 0.02	0.88 ± 0.05	0.89 ± 0.01	0.87 ± 0.02	0.90 ± 0.02
Total BFI (New bone/Total bone)	N/A	7.7 ± 3.6	9.2 ± 2.4	19.7 ± 6.1	23.4 ± 4.6	23.2 ± 2.0	29.5 ± 9.3

Table II shows the statistical effects for this data. There were no statistically significant differences between the suspended and control groups' cross-sectional areas ($P = 0.36$). However, the effect of time on the total CSA was statistically significant ($P < 0.001$) in both groups. HLS was found to have a statistically significant effect on the periosteal circularity values for the two groups, with the suspended groups humeri being more circular than the controls ($p < 0.01$). There was no statistically significant effect of time on the circularity values for the two groups ($P = 0.3$). There was a significant effect of time on the data with the BFI of both groups increasing significantly over the experimental period ($P < 0.001$). HLS caused no significant change in the total BFI of the suspended animals relative to the controls ($P = 0.17$).

groups. Table II shows the statistical effects for both time and HLS for these groups. There were no statistically significant differences between the suspended and control groups' cross-sectional areas ($P = 0.36$). However, the effect of time on the total CSA was statistically significant ($P < 0.001$) in both groups although it was not consistent over the full experimental period. HLS was found to have a statistically significant effect on the periosteal circularity values for the two groups, with the suspended groups humeri being more circular than the controls ($P < 0.01$). There was a significant effect of time on the data with the total BFI of both groups increasing significantly over the experimental period ($P < 0.001$). This was as expected with the BFI increasing as the animals grew. HLS caused no significant change in the total BFI of the suspended animals relative to the controls ($P = 0.17$).

In the femora, HLS resulted in a pronounced change in the mid-diaphyseal growth patterns. In order to illustrate these changes, graphs of each parameter against time are shown for both control and suspended groups (Figures 2–4). Although an increase was found in the total mid-diaphyseal cross-sectional area in both groups over time, a significant reduction in the CSA was found in the suspended group relative to the controls at all time points (Figure 2). HLS was found to have a statistically significant effect on the periosteal circularity values for the two groups (Figure 3), with the suspended groups femora being more circular than the controls ($P < 0.01$). Figure 4 shows the total BFI in the femora for the two groups. There was a significant effect of time on the data with the BFI of both groups increasing significantly over the experimental period ($P < 0.001$). This was as expected with the BFI increasing as the animals grew. There was a significant reduction in the total BFI of the suspended animals relative to the controls ($P < 0.001$) over the first 7 days of unloading which explains the reduction in the CSA that was found in the suspended group relative to the controls. However, after 7 days an increase in the total BFI was found in the suspended group relative to the controls. Figure 5 shows a mid-diaphyseal transverse cross-section of a rat femur viewed using UV epifluorescence, new bone formed can be seen as the bone lying outside the original periosteal surface labelled at day zero with oxytetracycline.

Table II. Statistical analysis of the effect of time and hindlimb suspension on humeral morphological parameters.

	Statistical effect of time	Statistical effect of HLS
Cross-sectional area (mm^2)	$\mathbf{P < 0.001}$	$P = 0.36$
Circularity $4\pi \times$ Area/[Perimeter]^2	$P = 0.3$	$\mathbf{P < 0.01}$
Total BFI (Total new bone/Total bone)	$\mathbf{P < 0.001}$	$P = 0.17$

Gene expression analysis

The DNA ladder of sequentially diluted cDNA samples demonstrated an ability to reproducibly detect close to 2-fold increases in gene expression (e.g. for GAPDH: 1.88 ± 0.53). In addition, a high correlation was found between the duplicate cDNA array values ($R^2 = 0.92$). Finally, no differences were found between individual candidate genes relative to the two housekeeping genes or between the triplicate SMART arrays. Figure 6 shows the osteocalcin/GAPDH versus time data for the control and suspended groups. A drop in the osteocalcin levels measured at 1 and 3 days was found in the suspended group. However, after 7 days there was a significant increase in the level of osteocalcin in relation to the control group. After this time the levels of osteocalcin expressed dropped off. A similar trend was also found for c-fos (Figure 7). This result might explain the bone forming response, which was observed after 7 days in the femora of the suspended animals (Figures 4 and 5).

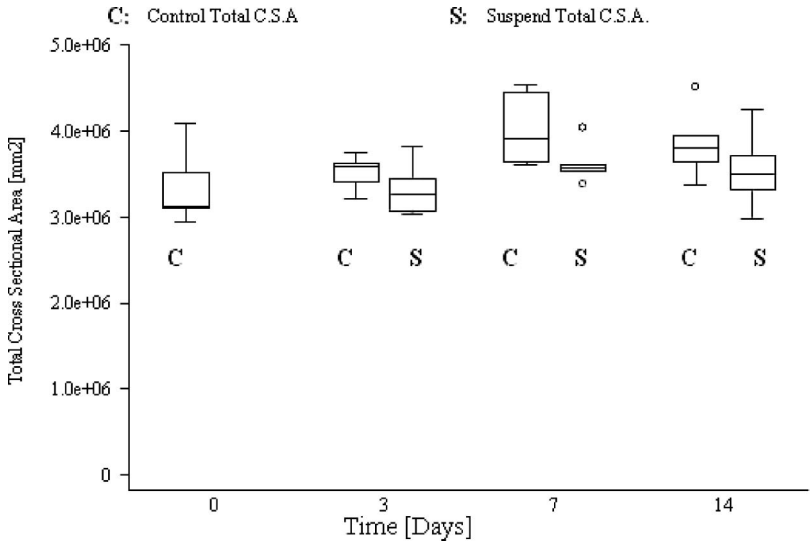

Figure 2. Femoral total mid-diaphyseal cross-sectional area versus time for control and suspended groups. An increase was found in the total mid-diaphyseal cross-sectional area in both groups over time but a significant reduction in the CSA was found in the suspended group relative to the controls at all time points.

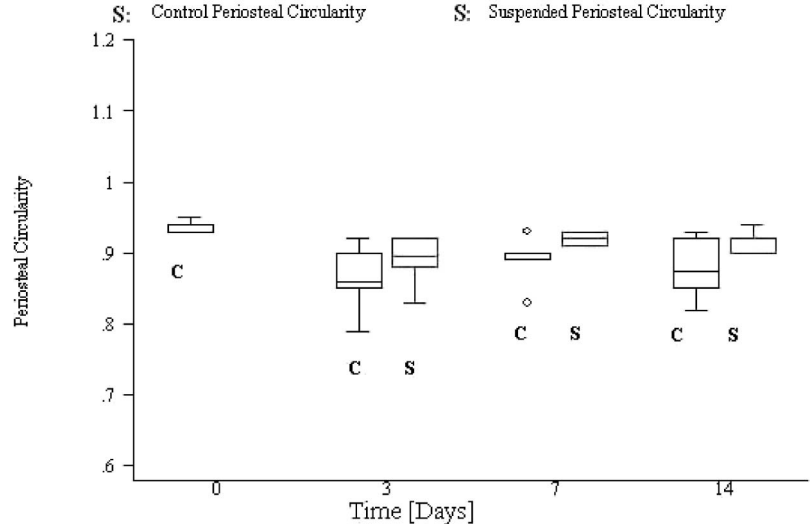

Figure 3. Femoral periosteal circularity versus time for control and suspended groups. HLS was found to have a statistically significant effect on the periosteal circularity values with the suspended groups femora being more circular than the controls ($P < 0.01$). There was no statistically significant effect of time on the circularity values for the two groups ($P = 0.26$).

Discussion

This study has shown that following hindlimb suspension, widespread changes take place in the unloaded bones of growing rats. Hindlimb suspension resulted in a significant reduction in the total mid-diaphyseal cross-sectional area in the femora of the suspended animals compared to controls. This effect of HLS was also evident in the reduced bone formation rates found in the suspended animals in the first 7 days of unloading. Therefore, it would appear that unloading elicits a coupled response of formation and resorption at periosteal and endosteal surfaces, respectively, although in growing animals the increased formation rates after 7 days in the suspended animals would indicate that periosteal formation is the predominant force over time in growing animals. This resumption in growth by the end of the second week of continuous suspension is consistent with results found by a number of other studies (van der Meulen et al. 1995; Vailas et al. 1988; Globus et al. 1984; Bikle et al. 1994a,b). In the humerii of the suspended animals, the primary response to the altered loading system in comparison to the controls was a change in bone shape with the humerii from the suspended animals bones becoming more circular at the periosteal surface relative to the controls. No increase in total mid-diaphyseal

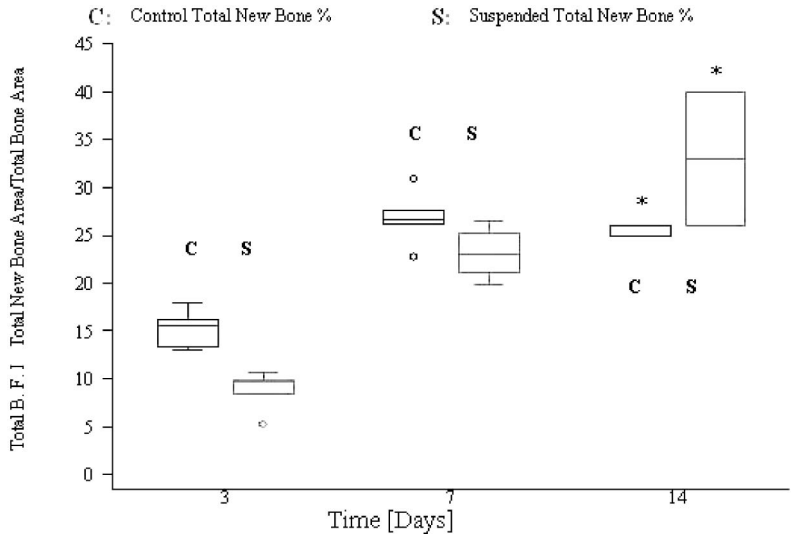

Figure 4. Femoral total bone formation index for control and suspended groups. The BFI of both groups increased significantly over the experimental period ($P < 0.001$). There was a significant reduction in the total BFI of the suspended animals relative to the controls ($P < 0.001$) over the first 7 days of unloading which explains the reduction in the CSA that was found in the suspended group relative to the controls. However, after 7 days an increase in the total BFI was found in the suspended group relative to the controls.

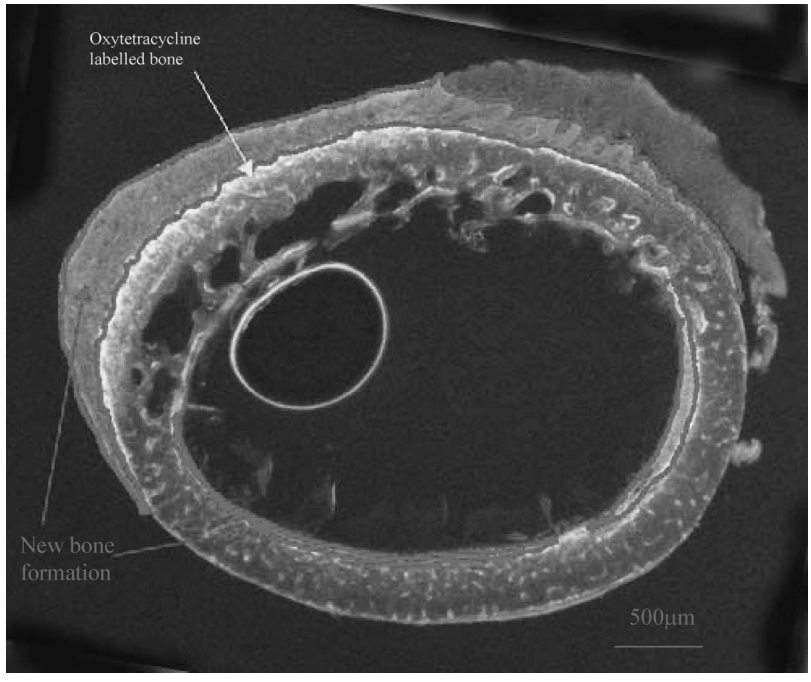

Figure 5. Mid-diaphyseal transverse cross-section of a rat femur viewed using UV epi-Fluorescence, new bone formed can be identified as the region periosteal bone formed outside the oxytetracycline label.

cross-sectional area or bone formation index of the humerii was found relative to the control animals. The CSA of both group's humerii increased in both groups over time although it was not consistent over the full experimental period. This was most likely simply due to the young animal's varying growth patterns.

A sensitive semi-quantitative method of gene expression analysis, involving the creation of SMART^{TM} cDNA arrays, was successfully implemented. Gene expression patterns of two candidate genes, c-fos and osteocalcin, were assessed in periosteal tissue. Altered gene expression patterns were identified and tracked over the suspension period with altered levels in osteocalcin and c-fos found consistent with the changes observed in the histological analysis. This system allowed the hypothesis of functional adaptation to be tested, i.e. that altered load results

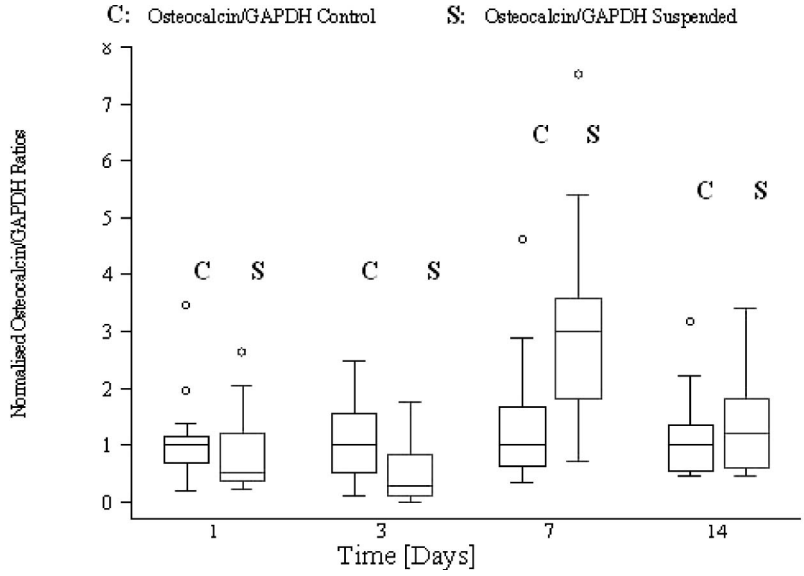

Figure 6. Normalised osteocalcin/GAPDH data versus time for the control and suspended groups. A drop in the osteocalcin levels measured at 1 and 3 days was found in the suspended group. However, after 7 days there was a significant increase in the level of osteocalcin in relation to the control group. After this time the levels of osteocalcin expressed dropped off.

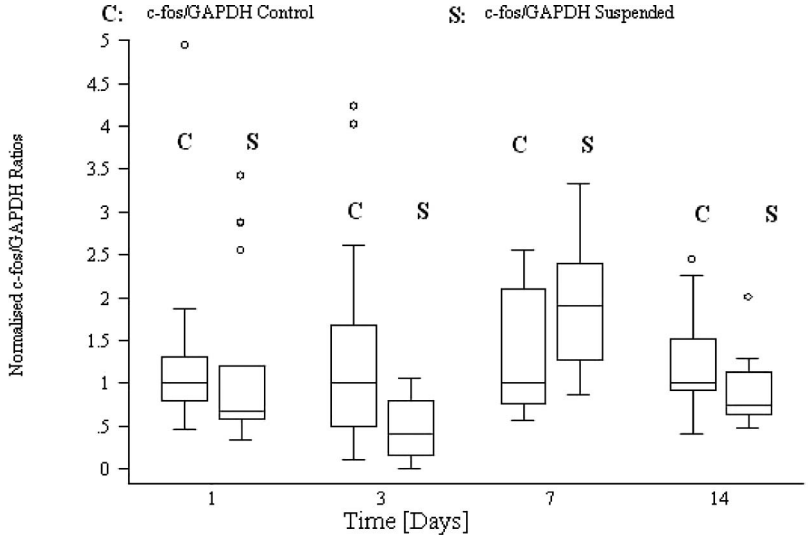

Figure 7. Normalised c-fos/GAPDH data versus time for the control and suspended groups. A drop in the c-fos levels measured at 1 and 3 days was found in the suspended group. However, after 7 days there was a significant increase in the level of c-fos in relation to the control group. After this time the levels of c-fos expressed dropped off.

in a drive for adaptation, which in turn results in altered gene expression patterns that alter the cellular phenotype and thus change in bone morphology and structural properties. In this experiment, cDNA arrays were created from the rat femoral periosteal samples collected post-mortem. The initial hypothesis was that HLS would result in a reduction in the expression of genes associated with bone formation. Although this hypothesis could not be verified in its entirety, there is enough evidence from the gene expression analysis to present some support for the hypothesis with both the reduction in c-fos and osteocalcin expression after both 1 and 3 days suspension consistent with the morphological analysis. Consequently, a quantitative method of gene expression analysis may be required to detect changes, and in particular reductions, in the gene expression levels of lowly expressed genes. In addition, more research with earlier and more frequent time increments might be necessary to fully validate the hypothesis. Following 7 days suspension a rise in the expression of c-fos and osteocalcin was detected in the suspended animals. These elevated levels are consistent with the bone formation response identified histologically where the BFI was found to be higher in the suspended femora relative to the controls after

14 days indicating that increased levels of formation were taking place in order to replace the bone which was lost in the first 7 days post-suspension. Previously, and in this study, HLS has been shown to result in a cessation/reduction in bone formation (van der Meulen et al. 1995; Globus et al. 1986; Bikle et al. 1994b). However as shown morphologically, this reduction in the rate of bone formation is transient with growth returning towards normal by 14 days of continued unloading. It is proposed therefore that the elevated levels of c-fos and osteocalcin found after 7 days in the periosteum are evidence of a return of the cells towards a state of normal growth. This reassertion of the growth response could be attributable to either systemic or genetic factors reasserting control in young fast growing rats.

In conclusion, this study has further demonstrated the use of HLS as a tool for monitoring the process of bone adaptation. The study monitored the changes that take place in the unloaded bones following HLS. These changes were tracked from gene expression changes in RNA extracted from periosteal tissue samples, to changes in the morphology of the adapted bone. These results indicate that changes in c-fos and osteocalcin expression are consistent with subsequent changes observed from the morphological analysis.

Acknowledgements

This work was supported by the Health Research Board of Ireland and the Royal College of Surgeons in Ireland.

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