DISRUPTION OF THE OSTEOCYTE CELLULAR NETWORK BY MICROCRACKS: A POSSIBLE STIMULUS FOR BONE ADAPTATION

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Abstract: Strenuous exercise undertaken by athletes, various diseases such as osteoporosis and osteopenia, or altered loading after joint replacements makes our bones become more susceptible to microdamage accumulation than those of normal human beings [1]. Experimental studies have shown that bone adaptation can be triggered by microdamage and may result in increased cellular activity. In this work, we investigated whether microcrack detection is related to rupturing of the cellular material itself due to crack face displacements. Using specific cell staining techniques, it was confirmed that relative crack displacements are capable of tearing cell processes between neighbouring osteocytes. No ruptured cell processes were found near the crack tip where the displacements are less. Rupturing of cell processes due to crack opening and shear displacement is a feasible new mechanism by which bone can detect and estimate the size of a microcrack. Ruptured cell processes may directly secrete passive and active components in the extracellular matrix, triggering a repair response.

Introduction

Both modelling and remodelling are processes by which changes in geometry, architecture and material properties (e.g. bone mass and density) occur in response to altered loading conditions and both contribute to bone adaptation [2-7]. It has led researchers to believe that the altered loading environment causes passive and active cellular responses by mechanotransduction. This is thought to cause altered gene and protein expression (biochemical signalling) [8-10]. Osteocytes with their fine cell-processes radiating from the cell, are believed to fulfil this function [3, 8, 11, 12]. It has been hypothesised that transmission of mechanical signals to the osteocyte skeleton via cell surface receptors [10] can occur directly through the solid matrix of the tissue due to load induced fluid flow [8, 13-16] as well as indirectly via fluid pressure and shear stresses [3, 17-20] through the lacuno-canalicular system. This network allows communication and transport of organic and inorganic matter between cells deep in the tissue and those located in the vicinity of vascular canals and bone surfaces. All this requires bone cells to be sensitive to mechanical and chemical stimuli.

Besides mechanical and fluid flow induced adaptation models, other models have been based on the idea that bone adaptation is controlled by the level of fatigue damage [21-25]. Fatigue damage, in the form of numerous microcracks, has been suggested to be targeted and act as a signal to regulate the bone adaptation process, since the level of damage provides a direct measure of the risk of failure [26-28]. If the damage, and particularly its rate of increase, is greater than can be repaired, failure will occur unless adaptation is initiated to reduce the stress levels. Frost [29] suggested that microdamage may trigger a remodelling response via disruption of the canalicular network surrounding the osteocytes. However, little is known of the means by which bone detects damage and 'decides' to initiate adaptation and what the damage / crack length threshold values are which trigger this cellular response.

We have proposed a theoretical model whereby osteocyte cell processes, spanning a crack, rupture due to relative crack-face opening and shear displacements [30]. These displacements were calculated using the theory of linear elastic fracture mechanics and thus an estimate regarding the number of broken cell-processes was made. At the maximum physiological stress [31, 32] a crack length of 91 μm was predicted, which is within the range of average crack lengths measured experimentally [33-38]. For a 100 μm crack (a typical crack length found in bone), processes begin to rupture at a stress of 28 MPa, which is somewhat lower than the normal maximum physiological stress [31, 39, 40]. Although the model was idealised, using homogeneous transverse isotropic material properties, a uniform distribution of osteocytes and cell processes, and a crack growth direction similar to the fibre direction in the bone but not considering the variability from one crack to another, this analysis demonstrated quite accurately that repair may be instigated by detecting the first ruptures of cell processes.

While this theoretical model predicted that cell-processes can be ruptured by crack-face displacements, no experimental data were available to confirm this. This paper discusses the experimental work carried out to investigate whether propagating cracks can rupture cell-processes *in vitro*. In order to assess this, cracks

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were grown in notched bone specimens. Histological sections and image analysis software were used to assess whether cellular material was damaged and how bone microstructure affects crack propagation. Furthermore, detailed finite element models were made of the tested specimens, including the crack geometry, to access whether linear elastic material properties could be used to estimate crack displacements.

Materials and Methods

Bone samples were taken from the mid-diaphyses of bovine femora and tibiae and immediately frozen (-20ºC). In total, 31 rectangular longitudinal specimens were cut, using a diamond saw cutting wheel (Struers Minitron, Copenhagen, Denmark). Notches were cut in two different orientations: I) Transverse notches and, II) Longitudinal notches. Specimens with longitudinal notches had a thickness of 1 mm and measured 20x20 mm, specimens with transverse notches had a thickness of 0.6mm and measured 35x15mm. The thickness values of 0.6 and 1 mm were found to work best to initiate stable crack growth: thicker sections made it more difficult to control crack growth and resulted in spontaneous cracking; thinner sections made it more difficult to create the notch.

Section preparation
Sections of bone were manually ground down between two pieces of silicon carbide paper (400 and 1200 grade successively) in a circular fashion using light pressure. Sections were then stained for 24 hours using alizarin ($0.5x10^{-3}$ M, fluorescent dye, Sigma Aldrich, UK) in a vacuum desiccator and stored in a freezer. Staining was used to obtain better contrast between the bone and the new formed crack. Two hours prior to testing, the samples were taken out of the freezer (-20ºC) and kept at 4ºC in saline, to prevent drying out.

Test rig
The rig design consisted of two grips, one attached to a load cell to monitor the forces applied during testing and one attached to a spindle in order to apply a load. A C-shaped frame measuring 165x68mm, having a thickness of 16 mm holds these components together. The load cell (KD 40S, ME-Meßsyteme Germany), measures tensile or compressive forces in the range of 0-2000N (sensitivity, ±0.1%). These measured forces were amplified (GSV-2, ME-Meßsyteme Germany) and directly read out to a PC. The spindle by which the loads are applied is attached to the second grip. Both grips slide in a longitudinal slot to prevent rotation and ensure uniaxial loading

In order to assess whether osteocyte cell processes had ruptured, two methods were used; Scanning Electron Microscopy (SEM) and confocal microscopy. Osteocytes and their lacunae are large enough to be easily identified using optical microscopes. However, the fine canaliculi, radiating from the osteocytes, are less easily seen. The major problem in visually observing canaliculi is that the spaces are too fine to be identified by merely staining the surrounding tissue. As such, high magnification (SEM) is required, or the use of a second fluorescent agent providing enough contrast between the canaliculi and the surrounding tissue (confocal microscopy).

Testing procedure
The rig with specimen was positioned under a UV epi-fluorescence microscope (100x mag., Nikon Eclipse E800) which was focused on the notch tip. By using UV light clearer and sharper images can be obtained between the fluorescent dye, alizarin, and the bone. The sample was loaded by rotating the nut; load was kept constant during the test. Images were recorded at set intervals (every 15 sec) and analysed using specialised image analysis software (Lucia Measurement version 4.7.1.). Crack propagation was stopped once the crack tip was outside the field of focus. The crack lengths were measured from the origin (notch) to the crack tip. The crack angles were measured with respect to the loading direction. Once the specimens had been tested, they were kept refrigerated at 4 ºC. Ten randomly chosen specimens (5 transverse notched and 5 longitudinal notched) were examined using a scanning electron microscope (Hitachi S-3500N). The remaining 21 specimens were used for confocal microscopy analysis.

The cell staining protocol
The bone samples were fixed on slides and washed three times in PBS (Phosphate Buffer Solution). Cell membranes were permeablised for 10 minutes using a PBS solution containing 0.2% Triton X100, followed by three washes. Phalloidin was used to stain filamentous actin (F-actin) for 45 minutes in a vacuum desiccator followed by three washes in PBS. Slides were mounted with Dako Fluorescent mounting medium (DAKO Corporation, CA, USA) under a cover slip and analysed using confocal microscopy (Zeiss LSM 510 META, Zeiss, Germany).

Results

Cracks were successfully propagated and monitored in all 31 specimens. Crack length varied from 0 to 891 µm for longitudinal notches and from 0 to 1345 µm for transverse notches with crack angles varying between 9.4 and 36.4 degrees to the loading direction. These cracks are well within the range of typical values found in bone [37, 38, 41, 42]. Of the 31 specimens, 4 cracks started kinking during the test. Kinking is the out-of-plane growth of a pre-existing straight crack. Kinked cracks appear if microstructural features deflect the crack from the primary growth direction, or when a crack was not formed in the primary growth direction (see Figure 1).

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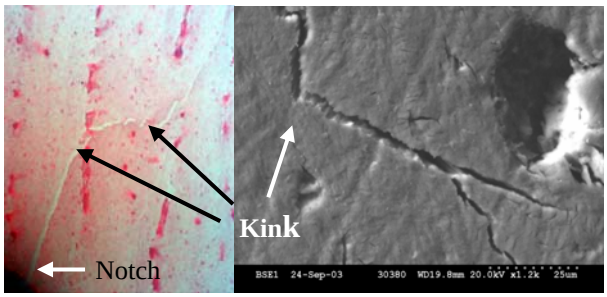

Figure 1: Illustration of kinked crack under UV-epifluorescence microscope (left) and SEM (right)

Scanning Electron Microscopy
Examination of the cracked specimens using SEM showed fibres crossing the crack faces near the crack tip. In regions where larger crack face displacements occurred, further from the crack tip, fibres were not found to cross the crack face, indicating complete rupture. Strands of organic matter were found to cross the crack, however no conclusions could be drawn as to whether these are collagen fibres or osteocytes cell processes. Furthermore, SEM examination can only view the surface: it does not provide insight into the three-dimensional distribution of cell-processes throughout the matrix.

Cell staining and confocal microscopy
In order to distinguish whether the fibres observed are comprised of collagen or if they are part of the cytoskeleton, specific staining techniques were applied. The combination of cell staining and confocal microscopy provides a non-destructive method for scanning through the depth of a specimen. Figure **2** shows a confocal microscopy image of a crack and three osteocytes (solid arrows).

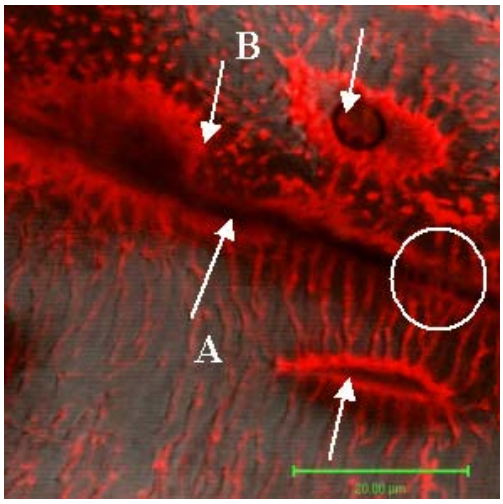

Figure 2: A confocal microscopy image of a crack and three osteocytes (solid arrows). The crack (A) has travelled through the lacuna of one osteocyte (B), which appears to have remained intact. The majority of the cell processes are broken by the crack, but some processes seem to cross the crack faces (circle)

The crack (A) has travelled through the lacuna of one osteocyte (B), which appears to have remained intact. However, the majority of the cell processes are broken by the crack, although some processes seem to cross the crack faces (circle). This pattern was seen in all slides examined. Near the origin of the crack (at the notch), all canaliculi were found to be broken, whereas near the crack tip the majority of the processes were still intact.

Discussion

In order to assess if crack face displacements can rupture cellular material a total of 31 specimens were analysed. Furthermore, a method was developed which does not require bone samples to be decalcified, allowing the use of a secondary fluorescent agent to create enough contrast to detect osteocyte cell processes near microcracks.

Observations were made of organic matter crossing the crack faces using confocal microscopy. Similar observations have been reported by other researchers [43-45], however they have failed to establish the nature of the organic matter crossing between the cracks faces. In this work we have shown that microcracks in bone cause damage to the cellular material, in the form of rupturing of cell processes due to crack opening and shear displacements by using specific cell stains. This damage to living material might act as a cellular transducing mechanism in bone. The number of broken cell processes may be a means by which bone is able to estimate the crack size and orchestrate its response either by remodelling or by bone deposition on the outer surface. Near the crack tips, cell processes remained intact and were observed spanning the crack. Crack face displacements in this region were clearly not sufficient to cause rupture of cell processes. This shows that not all cell processes break due to crack face displacements. The transition point between cell process rupture and the region where they remained intact could not be established precisely by the method used. This was due to constant changes in crack propagation rates, crack opening and crack shear displacements. As the propagation rate decreased, an increase in crack opening and shear displacement occurred. Once the crack continues to propagate, crack displacements decrease. This type of crack growth is typical of brittle materials and has been extensively described in the literature [46-48]. The mechanism, as proposed by Scott *et al*. [49], is stick-slip. Stick-slip occurs when sharp cracks under low stresses start to blunt as the stresses rise. When the crack radius is deformed to a relatively large extent, a new sharp crack suddenly develops at the crack tip and rapidly increases in length. Subsequently, the crack becomes dormant due to microstructural barriers. This process is repeated once stress levels increase again.
Recent data from You *et al*. [50] showed that the diameter of cell processes may vary between 50 to 410 nm and could be position dependent. In our previous analysis [30], failure criteria were set at 200 nm for

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crack opening or shear displacement. As such, it is difficult to estimate how much strain is required for process failure. However, the crack displacements were successfully predicted using linear elastic material properties, without taking the dynamic nature of a propagating crack into account. Better estimations were made for transverse notches than for longitudinal notches, which can be further improved if one takes the fibre direction into account.

The mechanism by which cell processes are broken due to crack opening and shear displacements may explain observations of osteocyte death. Process ruptures may interfere with nutrient and waste exchange and may alter the stimulus caused by fluid shear flow. Some scientists have proposed that osteocytes continuously send out an inhibitory signal, preventing osteoclastic activity [27, 51]. Our theory [30], regarding cell process rupture, which might induce osteocyte death (apoptosis/necrosis) or a signalling response (passive or active) may explain why Bentolila *et al.* [25] observed BMU activity in rats which had microdamage and why no remodelling was found in those that had no microdamage in their bone cortex. However, to what extent this affects the osteocyte-canalicular network and at what distance from the source of the trauma remains unknown. In other words, if rupture of cell processes triggers a response (chemically, or by necrosis/ apoptosis of osteocytes), how is this 'message' distributed through the cellular network? Mechanotransduction may not be necessary if ruptured cell processes, due to relative crack face displacements, provide the stimulus for bone adaptation. Ruptured cell processes secreting active and passive compounds (e.g. cytokines and growth factors [52-54]) in the extracellular matrix, which could diffuse through the matrix, may trigger a cellular response of the osteoblasts and osteoclasts. The existing mechanotransduction theory assumes that bone responds to altered load, causing altered gene expression followed by alterations in protein expression. Several proteins, linked to cell damage repair have been identified, however that they might be released directly in the extracellular matrix after rupture has not been considered. This mechanism looks very plausible since osteocytes contain numerous cell processes, suggesting that damage to the processes occurs on a regular basis. Signal transmission through direct cell-to-cell contact or by diffusion provides a much simpler mechanism to regulate bone adaptation compared to the existing mechanotransduction theory.

As part of an ongoing project, further research will be conducted in order to quantify the effects of ruptured cell processes and to determine how many broken cell processes are required to surpass the response threshold. This might provide more insight into whether ruptured cell processes cause osteocyte death and what alterations in protein expression are triggered.

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References

[1] STROMSOE, K. (2004): 'Fracture fixation problems in osteoporosis', *Injury*, 35: 107-113.
[2] BURR, D.B., A.G. ROBLING AND C.H. TURNER (2002): 'Effects of biomechanical stress on bones in animals', *Bone*, 30: 781-786.
[3] COWIN, S.C., L. MOSS-SALENTIJN AND M.L. MOSS (1991): 'Candidates for the mechanosensory system in bone', *J Biomech Eng*, 113: 191-197.
[4] FORWOOD, M.R., I. OWAN, Y. TAKANO, AND C.H. TURNER (1996): 'Increased bone formation in rat tibiae after a single short period of dynamic loading in vivo', *Am J Physiol*, 270: E419-423.
[5] HSIEH, Y.F. AND C.H. TURNER (2001): 'Effects of loading frequency on mechanically induced bone formation', *J Bone Miner Res*, 16: 918-924.
[6] MOSLEY, J.R. AND L.E. LANYON (1998): 'Strain rate as a controlling influence on adaptive modeling in response to dynamic loading of the ulna in growing male rats', *Bone*, 23: 313-318.
[7] LEE, T.C., A. STAINES AND D. TAYLOR (2002): 'Bone adaptation to load: microdamage as a stimulus for bone remodelling', *J Anat*, 201: 437-446.
[8] BURGER, E.H. AND J. KLEIN-NULEND (1999): 'Mechanotransduction in bone: role of the lacuno-canalicular network', *Faseb J*, 13 Suppl: S101-112.
[9] YOU, J., G.C. REILLY, X. ZHEN, C.E. YELLOWLEY, Q. CHEN, H.J. DONAHUE, AND C.R. JACOBS (2001): 'Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts', *J Biol Chem*, 276: 13365-13371.
[10] WANG, N., J.P. BUTLER AND D.E. INGBER (1993): 'Mechanotransduction across the cell surface and through the cytoskeleton', *Science*, 260: 1124-1127.
[11] COWIN, S.C. (2002): 'Mechanosensation and fluid transport in living bone', *J Musculoskel Neuron Interact*, 2: 256-260.
[12] AARDEN, E.M., E.H. BURGER AND P.J. NIJWEIDE (1994): 'Function of osteocytes in bone', *J Cell Biochem*, 55: 287-299.
[13] CARTER, D.R., D.P. FYHRIE AND R.T. WHALEN (1987): 'Trabecular bone density and loading history: regulation of connective tissue biology by mechanical energy', *J Biomech*, 20: 785-794.
[14] HUISKES, R. AND S.J. HOLLISTER (1993): 'From structure to process, from organ to cell: recent developments of FE-analysis in orthopaedic biomechanics', *J Biomech Eng*, 115: 520-527.

[15] BURR, D.B., C. MILGROM, R.D. BOYD, W.L. HIGGINS, G. ROBIN, AND E.L. RADIN (1990): 'Experimental stress fractures of the tibia. Biological and mechanical aetiology in rabbits', *J Bone Joint Surg Br*, 72: 370-375.

[16] FROST, H.M. (1987): 'Bone "mass" and the "mechanostat": a proposal', *Anat Rec*, 219: 1-9.

[17] WEINBAUM, S., S.C. COWIN AND Y. ZENG (1994): 'A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses', *J Biomech*, 27: 339-360.

[18] DUNCAN, R.L. AND C.H. TURNER (1995): 'Mechanotransduction and the functional response of bone to mechanical strain', *Calcif Tissue Int*, 57: 344-358.

[19] FORWOOD, M.R. AND C.H. TURNER (1995): 'Skeletal adaptations to mechanical usage: results from tibial loading studies in rats', *Bone*, 17: 197S-205S.

[20] TURNER, C.H. AND M.R. FORWOOD (1994): 'On animal models for studying bone adaptation', *Calcif Tissue Int*, 55: 316-317; author reply 318.

[21] BURR, D.B., M.R. FORWOOD, D.P. FYHRIE, R.B. MARTIN, M.B. SCHAFFLER, AND C.H. TURNER (1997): 'Bone microdamage and skeletal fragility in osteoporotic and stress fractures', *J Bone Miner Res*, 12: 6-15.

[22] VICECONTI, M. AND A. SEIREG (1990): 'A generalized procedure for predicting bone mass regulation by mechanical strain', *Calcif Tissue Int*, 47: 296-301.

[23] PRENDERGAST, P.J. AND D. TAYLOR (1994): 'Prediction of bone adaptation using damage accumulation', *J Biomech*, 27: 1067-1076.

[24] MARTIN, R.B., S.M. STOVER, V.A. GIBSON, J.C. GIBELING, AND L.V. GRIFFIN (1996): 'In vitro fatigue behaviour of the equine third metacarpus: remodeling and microcrack damage analysis', *J Orthop Res*, 14: 794-801.

[25] BENTOLILA, V., T.M. BOYCE, D.P. FYHRIE, R. DRUMB, T.M. SKERRY, AND M.B. SCHAFFLER (1998): 'Intracortical remodeling in adult rat long bones after fatigue loading', *Bone*, 23: 275-281.

[26] TAYLOR, D. (1997): 'Bone maintenance and remodeling: a control system based on fatigue damage', *J Orthop Res*, 15: 601-606.

[27] MARTIN, R.B. (2000): 'Toward a unifying theory of bone remodeling', *Bone*, 26: 1-6.

[28] PARFITT, A.M. (2002): 'Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression', *Bone*, 30: 5-7.

[29] FROST, H.M., *Presence of microscopic cracks in vivo in bone*, in *Henry Ford Hosp Bull*. 1960. p. 25-35.

[30] TAYLOR, D., J.G. HAZENBERG AND T.C. LEE (2003): 'The cellular transducer in damage-stimulated bone remodelling: a theoretical investigation using fracture mechanics', *J Theor Biol*, 225: 65-75.

[31] MILGROM, C., A. FINESTONE, Y. LEVI, A. SIMKIN, I. EKENMAN, S. MENDELSON, M. MILLGRAM, M. NYSKA, N. BENJUYA, AND D. BURR (2000): 'Do high impact exercises produce higher tibial strains than running?' *Br J Sports Med*, 34: 195-199.

[32] MILGROM, C., A. FINESTONE, A. SIMKIN, I. EKENMAN, S. MENDELSON, M. MILLGRAM, M. NYSKA, E. LARSSON, AND D. BURR (2000): 'In-vivo strain measurements to evaluate the strengthening potential of exercises on the tibial bone', *J Bone Joint Surg Br*, 82: 591-594.

[33] BURR, D.B. AND T. STAFFORD (1990): 'Validity of the bulk-staining technique to separate artifactual from in vivo bone microdamage', *Clin Orthop*: 305-308.

[34] BURR, D.B. AND R.B. MARTIN (1993): 'Calculating the probability that microcracks initiate resorption spaces', *J Biomech*, 26: 613-616.

[35] LEE, T.C., E.R. MYERS AND W.C. HAYES (1998): 'Fluorescence-aided detection of microdamage in compact bone', *J Anat*, 193 (Pt 2): 179-184.

[36] MORI, S. AND D.B. BURR (1993): 'Increased intracortical remodeling following fatigue damage', *Bone*, 14: 103-109.

[37] LEE, T.C., S. MOHSIN, D. TAYLOR, R. PARKESH, T. GUNNLAUGSSON, F.J. O'BRIEN, M. GIEHL, AND W. GOWIN (2003): 'Detecting microdamage in bone', *J Anat*, 203: 161-172.

[38] O'BRIEN, F.J., D. TAYLOR, G.R. DICKSON, AND T.C. LEE (2000): 'Visualisation of three-dimensional microcracks in compact bone', *J Anat*, 197 Pt 3: 413-420.

[39] RUBIN, C.T. AND L.E. LANYON (1982): 'Limb mechanics as a function of speed and gait: a study of functional strains in the radius and tibia of horse and dog', *J Exp Biol*, 101: 187-211.

[40] NUNAMAKER, D.M., D.M. BUTTERWECK AND M.T. PROVOST (1990): 'Fatigue fractures in thoroughbred racehorses: relationships with age, peak bone strain, and training', *J Orthop Res*, 8: 604-611.

[41] MARTIN, R.B., D.B. BURR AND N.A. SHARKEY. Skeletal Tissue Mechanics. Berlin, Springer Verlag.1998.

[42] FAZZALARI, N.L., M.R. FORWOOD, K. SMITH, B.A. MANTHEY, AND P. HERREEN (1998): 'Assessment of cancellous bone quality in severe osteoarthrosis: bone mineral density, mechanics, and microdamage', *Bone*, 22: 381-388.

[43] NALLA, R.K., J.H. KINNEY AND R.O. RITCHIE (2003): 'Mechanistic fracture criteria for the failure of human cortical bone', *Nat Mater*, 2: 164-168.

[44] NALLA, R.K., J.J. KRUZIC AND R.O. RITCHIE (2004): 'On the origin of the toughness of mineralized tissue: microcracking or crack bridging?' *Bone*, 34: 790-798.

[45] NALLA, R.K., J.J. KRUZIC, J.H. KINNEY, AND R.O. RITCHIE (2005): 'Mechanistic aspects of fracture and R-curve behavior in human cortical bone', *Biomaterials*, 26: 217-231.

[46] MARTIN, R.H. (1997): 'Delamination characterization of woven glass/polyester

ISSN: 1727-1983

composites.' *Journal of Composite Technological Research*, 19: 20-28.

[47] EBELING, T., A. HILTNER AND E. BAER (1997): 'Delamination failure of a woven glass fiber composite', *Journal of Composite Materials*, 31: 1318-1333.

[48] SUZUKI, Y., Z. MAEKAWA, H. HAMADA, A. YOKOYAMA, T. SUGIHARA, AND M. HOJO (1993): 'Influence of silane coupling agents on interlaminar fracture in glass fibre fabric reinforced unsaturated polyester laminates.' *J Mater Sci*, 28: 1725-1732.

[49] SCOTT, J.M., G.M. WELLS AND D.C. PHILLIPS (1980): 'Low temperature crack propagation in an epoxide resin', *Journal of Matererial Science*, 15: 1436-1448.

[50] YOU, L.D., S. WEINBAUM, S.C. COWIN, AND M.B. SCHAFFLER (2004): 'Ultrastructure of the osteocyte process and its pericellular matrix', *Anat Rec*, 278A: 505-513.

[51] MAROTTI, G., M. FERRETTI, M.A. MUGLIA, C. PALUMBO, AND S. PALAZZINI (1992): 'A quantitative evaluation of osteoblast-osteocyte relationships on growing endosteal surface of rabbit tibiae', *Bone*, 13: 363-368.

[52] JONES, J.I., M.E. DOERR AND D.R. CLEMMONS (1995): 'Cell migration: interactions among integrins, IGFs and IGFBPs', *Prog Growth Factor Res*, 6: 319-327.

[53] WILSON, S.E., R.R. MOHAN, M. NETTO, V. PEREZ, D. POSSIN, J. HUANG, R. KWON, A. ALEKSEEV, AND J.P. RODRIGUEZ-PEREZ (2004): 'RANK, RANKL, OPG, and M-CSF expression in stromal cells during corneal wound healing', *Invest Ophthalmol Vis Sci*, 45: 2201-2211.

[54] DOXEY, D.L., C.W. CUTLER AND A.M. IACOPINO (1998): 'Diabetes prevents periodontitis-induced increases in gingival platelet derived growth factor-B and interleukin 1-beta in a rat model', *J Periodontol*, 69: 113-119.

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