2.5. The Effect of Fluoride on Osteocalcin Levels

Dandona *et al.* (1988) administered NaF to healthy male subjects for three weeks to test whether the administration of NaF *in vivo* had an effect on osteocalcin concentration. The mean osteocalcin at three weeks was significantly higher than the pre-treatment mean. Six weeks after the cessation of fluoride treatment, the mean serum osteocalcin concentration had returned to the pre-treatment baseline. They concluded that fluoride in normal subjects over a short period increases serum osteocalcin and probably stimulates osteoblastic activity.

Srivastava *et al.* (1989) analyzed sera from patients with skeletal fluorosis and showed for the first time that patients with fluorosis have markedly elevated osteocalcin. This was evident even in the presence of subnormal concentrations of clinical vitamin D and calcium. This is relevant because it has previously been shown that $1,25(OH)_2D$ (the activated form of vitamin D) stimulates osteocalcin production by *in vitro* osteoblast cultures, that clinical vitamin D deficiency may prevent an increase in osteocalcin concentrations in response to parathyroid hypersecretion, and that the administration of $1,25(OH)_2D$ stimulates osteocalcin *in vivo* (Zerwekh et al., 1985). The stimulatory effect of fluoride on osteocalcin production in patients with skeletal fluorosis would, therefore, appear to be greater than the inhibitory effect of vitamin D deficiency.

In a study investigating the value of slow release sodium fluoride treatment in the management of human osteoporosis, Pak *et al.* (1989) found that sodium fluoride therapy increases serum osteocalcin concentrations in osteoporotic patients and that serum osteocalcin concentrations correlated positively with the duration of treatment.

The evolution of bone changes induced by fluoride after the end of exposure was investigated in lambs by Chavassieux *et al.,* (1991). Along with an increase in bone formation it was found that serum osteocalcin was twice as high in treated animals compared with controls. In a separate study, the same investigators used a different sample group of ewes in which the early effects of two doses of sodium fluoride on bone remodelling were investigated.

One group of ewes received 1mg NaF/kg/day whilst the other group received a five-fold greater dose. Bone fluoride content significantly increased in the latter group along with osteoid perimeter and serum osteocalcin values (Chavassieux *et al.,*1991). In a later study investigating the effects of sodium fluoride on human osteoblastic cultures, however, Chavassieux *et al.* (1993) found that the short term increase in osteocalcin expression by these cells is reversed upon longer exposure of the culture to this anabolic agent over four months.

The effect of mitogenic doses of sodium fluoride on osteocalcin in the rat model was investigated by Ohta *et al.,* (1995), who found that serum osteocalcin levels were significantly increased together with mineral apposition rate and bone formation rate in fluoride-treated rats as opposed to controls.

2.6. Osteocalcin and Tooth Movement

While the study of bone biology using cultured cells has proved useful, inherent artefacts in the *in vitro* system limit extrapolation to the *in vivo* system. This is due to the difficulty in recapitulating the sequence of differentiation events in intact bone during exposure to environmental osteogenic stimuli, such as hormones, growth factors or mechanical stress. Hence, the animal model of orthodontically-facilitated tooth movement has provided a means by which the dynamics of bone remodelling can be more accurately investigated.

The orthodontic movement of murine teeth was used by Pavlin and Gluhak-Heinrich (2001) to elucidate the response of osteocalcin to mechanical stimuli over a period of 14 days. After 24 hours of applied force, there was an inhibition of osteocalcin expression by 80%, followed by a marked four-fold induction at day two. Osteocalcin expression peaked six-fold at day four, then fell. However, the levels remained over four-fold higher compared with controls at day six. These results suggested a relatively early expression of osteocalcin in differentiating osteoblasts.

Hence, this mouse osseoinductive model was found to provide direct *in vivo* evidence that the osteocalcin gene is mechanically induced in osteoblasts much earlier than the mineralization stage, since its high expression can be followed from the early stages of matrix deposition (two days post-stimulation), on the surface of the newly deposited osteoid, as well as in the cells deeply embedded in and surrounded by osteoid.

Interestingly, the distribution of high osteocalcin-expressing cells across the thickness of new osteoid indicates higher levels of synthesis on the surface and in the middle portion of the osteoid than at the osteoid/bone junction. This refutes the idea of exclusive osteocalcin expression in mature osteoblasts, *i.e.* in a late stage of differentiation.

In the light of the results from osteocalcin-deficient mice and a proposed suppressive role of osteocalcin in bone formation (Ducy *et al.,* 1996), its up-regulation in the areas of active deposition of bone matrix in the mouse orthodontic model is consistent with its possible role as a determinant of the rate of mechanically induced bone remodelling. Furthermore, Pavlin *et al.,* (2001) showed that the temporal progression of the mechanically-induced osteoblast phenotype in the murine model occurs very rapidly. This suggests that physiologically relevant mechanical osteoinductive signals *in vivo* are targeting a population of committed osteoblast precursor cells that are capable of rapidly responding by entering a differentiation pathway and initiating an anabolic skeletal adaptation process.

There are also significant differences in the genetic responses of cementoblasts and osteoblasts to mechanical stimulation.

Pavlin *et al.* (2001) found that there was only a moderate stimulatory treatment effect on the expression of osteocalcin expression in cementoblasts (similar induction of osteocalcin expression in both cementoblasts of acellular cementum and cementoblasts of cellular cementum), compared to a six-fold induction in osteoblasts.

Local administration of 1, 25-dihydroxyvitamin D_3, or prostaglandin E_1 has been shown to accelerate experimental tooth movement (Collins and Sinclair, 1988; Yamasaki *et al.*, 1980). Both chemicals act as potent inducers of bone resorption *in vitro* and *in vivo*. Repeated injections of prostaglandin E_1 in rat alveolar bone produce marked changes in bone morphology with increased resorption at the site where the drug was infiltrated. Administration of 1, 25-dihydroxyvitamin D_3 has been reported to induce an increase in enlarged osteoclasts on the bone surface.

In contrast, local osteocalcin administration in the rat model has no appreciable effect on the bone surface (Kobayashi *et al.*, 1996). Osteocalcin was found to induce mature osteoclasts on the pressure side of orthodontic tooth movement, not on the tension side. This suggested that osteocalcin has an additive effect on the rate of orthodontic tooth movement through the enhancement of orthodontic tooth movement on the pressure side.

In a similar experiment involving the local injection of osteocalcin adjacent to rat molars over a longer period of time, Hashimoto *et al.* (2001) came to the same conclusion.

They suggested that osteocalcin might act as a chemoattractant for osteoclast precursor cells and an enhancer of maturation of multinuclear osteoclasts on the pressure side, where it may act in concert with bone resorbing factors (such as macrophage colony-stimulating factor [M-CSF]).

This then gives rise to an appropriate microenvironment for osteoclastogenesis. It was shown that there was acceleration of tooth movement following local application of osteocalcin in the early experimental period. Histological examination revealed that this acceleration was due to enhanced recruitment of osteoclasts. In contrast to the early days of the experiment, osteocalcin did not show an additive effect on the rate of orthodontic tooth movement towards the end of the ten day experimental period.

Previously, it has been shown that the chemotactic activity of osteocalcin for human peripheral monocytes (osteoclast precursor cells) displayed a bell-shaped dose-response curve. In addition, the serum osteocalcin level increased in the later phase of tooth movement in rats (King and Keeling, 1995). It is therefore possible that the increase in serum osteocalcin associated with tooth movement may diminish the effect of exogenous osteocalcin in the later period of tooth movement (Hashimoto *et al.*, 2001).

King and Keeling (1995) examined alveolar bone turnover and orthodontic tooth movement following appliance decay using closed coil springs in the rat model.

They observed that 93% of appliance activity was lost at day 16, at which time a second wave of bone remodelling began, with abrupt inhibition of ongoing bone formation and the initiation of a second wave of bone resorption. This was accompanied by a parallel rise in the amount of serum osteocalcin detected. The spike in osteocalcin values reached a nadir at day 22 when experimentation was concluded. They interpreted this data as being predictive of a second wave of bone formation (which did not eventuate due to premature conclusion of the experiment) rather than being representative of the occurring resorption.

2.7. Orthodontic Force Systems

2.7.1 Duration of Force

Frequency and duration of force application are known to be significant characteristics of the mechanical stress stimulating bone remodelling. Reitan (1951) reported on movement of dog incisors when application of force was discontinued for 20 seconds every ten minutes or for two minutes every two hours over 36 hours and stated that the amount of tooth movement was the same with continuous force. Gibson *et al.* (1992) reported that only one hour of force application was effective for mesial movement of rat molars.

In contrast, Konoo (1988) reported that force application of one hour per day for 14 days was not effective in moving molars in rats. Davidovitch and Shanfeld (1975) demonstrated that at least four hours of continuous force was required to increase the level of cyclic nucleoside that stimulated cell specialization.

Proffit (1999) insisted that there was a threshold of duration of force application for initiating clinical tooth movement. He also suggested that a loading time of more than six hours was effective for tooth movement and that a 24-hour, continuous force was optimal.

In all of these aforementioned studies, various force magnitudes, durations, and methods were used but the precise relationship between force duration and tooth movement remain unresolved. On the other hand, with substantially different forces, rates of tooth movement may be almost the same among or within individuals (Steiner *et al.*,1981).

These individual differences in tooth movement characteristics are possibly related to individual variation in the structure of and cellular activity within the periodontal ligament and alveolar bone (Steigman and Michaeli, 1981) or to localized differences in the expression of factors such as cytokines and growth factors (Bridges *et al.,* 1981).

Interrupted orthodontic forces have also been shown to produce tooth movement similar to that generated by continuous forces with less damage to the structures of the periodontal ligament (Gibson *et al.,* 1992). In a series of experiments, King and his associates tested the relationship between force magnitude, duration, and frequency. Several conclusions emerged that supported previous research findings.

Light short-term or cyclic force applications can alter tooth position in a way comparable to light continuous forces. Significant amounts of bone turnover and remodelling events consistent with the direction of loading, and associated with tooth movement, continue for an indeterminate period after appliance decay, or appliance removal (King *et al.,* 1995).

An interesting finding in a diurnal orthodontic rat study by Igarashi *et al.* (1998) was that there was no significant difference in tooth movement between the light-period group and the whole-day group. This infers that the intermittent application of force for 12 hours (07:00 to 19:00) can produce tooth movement comparable to that produced by continuous force in rats. Previous studies both in animals (Gibson *et al.,* 1992) and human beings (Stutzmann and Petrovic, 1984) have shown that the application of intermittent or short-term forces for less than 24 hours are as effective as, or even more effective than continuous orthodontic force. However, it is possible that this might only be true when the force is applied at certain times of the day (during resting periods).

2.7.2 Generation of a Constant Orthodontic Force

Efficient, biological tooth movement by means of light continuous forces is the preferred treatment modality in contemporary orthodontics. In the application of orthodontic force, consideration of the maximum force of the appliance and the load-deflection rate of the deactivation curve are of paramount importance.

2.7.2.1 Superelastic Coil Springs

To this end, Von Fraunhofer *et al.* (1993) compared the force delivery by NiTi coil springs to that provided by comparable stainless steel springs and found that the former delivered optimal forces for orthodontic tooth movement over a longer activation range than the latter. Superelastic springs also offer superiority to other materials in that they are resistant to permanent deformation Superelastic coil springs are able to deliver a constant force over a long range by virtue of the crystalline transition between austenite to martensite. This property in combination with the associated low stiffness and large springback is desirable in the choice of a model for orthodontic movement (Melsen *et al.*,1994).

Two previous clinical studies investigated the efficiency and consistency of space closure with elastic modules compared to 100g, 150g and 200g Sentalloy nickel-titanium closed coil springs (Samuels *et al.,* 1993; Samuels *et al.,* 1998).

These studies both concluded that these springs produced more consistent space closure than elastic modules. A more recent randomized clinical trial conducted by Dixon *et al.* (2002) confirmed this finding, showing that superelastic NiTi springs had the advantage of giving significantly quicker and more consistent rates of space closure than elastic chain or active modules.

In a study of force systems developed from coil springs supplied by different manufacturers, Melsen *et al.* (1994) found that only the Sentalloy coil springs from GAC exhibited the behaviour of superelastic wire. In a study of the force characteristics of these same GAC superelastic closed coil springs, Tripolt *et al.* (1999) determined that the light springs (100g) delivered a more constant force during unloading than the medium (150g) and heavy springs (200g).

3. References

Ajie HO, Hauschka PV, Kaplan IR, Sobel H. Comparison of bone collagen and osteocalcin for determination of radiocarbon ages and paleodietary reconstruction. Earth Plan Sc Lett 1991; 107:380-388.

Amman P, Rizzoli R, Caverzasio J, Bonjour J-P. Fluoride Potentiates the Osteogenic Effects of IGF-I in Aged Ovariectomized Rats. Bone 1998; 22:39-43.

Arnett, TR. Update on Bone Biology. Eur J Orthod 1990; 12: 81-90.

Ashizawa Y, Sahara N. Quantitative evaluation of newly formed bone in the alveolar wall surrounding the root during the initial stage of experimental tooth movement in the rat. Arch Oral Biol 1998; 43: 473-484.

Balena, R, Kleerekoper M, Foldes JA, Shih M-S, Sudhaker D, Rao HC, Schober HC, Parfitt AM. Effects of different regimens of sodium fluoride treatment for osteoporosis on the structure, remodeling and mineralization of bone. Osteoporos Int. 1998; 8: 428-435.

Baron R, Tross R, Vignery A. Evidence of sequential remodelling in rat trabecular bone: morphology of dynamic histomorphometry and changes during skeletal maturation. Anat Rec. 1984; 208: 137-145.

Baron R. Molecular mechanisms of bone resorption by the osteoclast. Anat Rec. 1989; 224: 317-24.

Bar-Shavit Z, Teitelbaum P, Reitsma A, Hall LE. Induction of monocyte differentiation and bone resorption by 1, 25-$(OH)_2$ D_3 . Proc Natl Acad Sci. USA. 1983; 80: 5907-5911.

Bartold PM, Miki Y, Narayanan AS, Page RC. Glycosaminoglycans of human cementum. J Perio Res 1988;23:13-17.

Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. Eur J Orthod 1997; 19: 615-621.

Baumgrass R, Williamson MK, Price PA. Identification of peptide fragments generated by digestion of bovine and human osteocalcin with the lysosomal proteinases cathepsin B, D, L, H and S. J Bone Miner Res. 1997; 12: 447-455.

Baylink D, Werdegal J, Stauffer M, Rich C. Effects of fluoride on bone formation, mineralization, and resorption in the rat.(1970) In: Vischer TL (ed) Fluorine in Medicine. Hans Huber, Bern, pp. 37-69.

Baylink DJ Farley JR, Lau KHW Wergedal J. Sodium fluoride does not increase human bone cell proliferation or protein synthesis in vitro. Calc Tiss Int. 1992; 50: 96-104.

Bellows CG, Heersche JN, Aubin JE. The effects of fluoride on osteoblast progenitors in vitro. J Bone Min Res 1991; 5 (Suppl.1): S101-S105.

Benyahu D, Shamay A, Wientroub S. Osteocalcin (BGP) gene expression, and protein production by marrow stromal adipocytes. Biochem Biophy Res Commun 1997; 231: 442-446.

Beresford J, Gallagher JA, Poser JW, Russell RGG. Production of osteocalcin by human bone cells in vitro. Effects of 1,25-$(OH)_2$ D3, 24,25-$(OH)_2$ D3, parathyroid hormone, and glucocorticoids. Metabolic Bone Disease and Related Research 1984; 5: 229-234.

Berkovitz BKB, Holland GR and Moxham BJ. A colour atlas and textbook of oral anatomy, histology and embryology 2nd edn. Mosby-Wolfe 1992.

Blumsohn A, Hannon RA, Eastell R. Apparent instability of osteocalcin in serum as measured with different commercially available immunoassays.
Clin Chem 1995; 41: 318-9.

Bodine PV, Komm BS. Evidence that conditionally immortalized human osteoblasts express an osteocalcin receptor. Bone 1999; 25: 535-543.

Bonewald LF, Kester MB, Schwartz Z, et al. Effects of combining transforming growth factor and 1,25 dihydroxyvitamin D3 on differentiation of human osteosarcoma (MG-63). Journal of Biological Chemistry 267: 8943-8949; 1992.

Bonjour JP, Caverzasio J, Rizzoli R. Effect of fluoride on bone cells. Res Clin Forums 1993; 15: 9-12.

Bosshardt SS, Schroeder HE. Cementogenesis reviewed: a comparison between human premolars and rodent molars. Anat Rec 1996; 245: 267-292.

Bosshardt DD, Masseredjian V, Nanci A. Root resorption and tissue repair in orthodontically treated human premolars. In: Davidovitch Z, Mah J, eds. Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants. Boston, Mass: Harvard Society for the Advancement of Orthodontics;1998:425-437.

Bosshardt DD and Selvig KA. Dental cementum: the dynamic tissue covering of the root. Periodontol 2000 1997; 13: 41-75.

Brezniak N and Wasserstein A. Orthodontically induced inflammatory root resorption. Part I: The basic science aspects. Angle Orthod 2002;72: 175-179.

Briancon D, Meunier PJ. Treatment of osteoporosis with fluoride, calcium, and vitamin D. Orthop Clin North Am 1981; 12: 629-647.

Bridges T, King G, Mohammed A. Effect of age on tooth movement and mineral density in alveolar tissues of the rat. Am J Orhod Dentofac Orthop1988; 93: 245 – 250.

Bronckers AL, Gay S, Finkelman RD, Butler WT. Developmental appearance of Gla-proteins (osteocalcin) and alkaline phosphatase in tooth germs and bones of the rat. Bone & Mineral 1987; 2: 361-373.

Bronckers ALJJ, Farach-Carson MC, Van Waveren E, Butler WT. Immunolocalization of Osteopontin, Osteocalcin, and Dentine Sialoprotein during dental root formation and early cementogenesis in the rat. J Bone Miner Res. 1994; 9: 833-841.

Bronckers ALJJ, Price PA, Schrijivers A, Bervoets TJM, Karsenty G. Studies of osteocalcin function in dentine formation in rodent teeth. Eur J Oral Sci 1998; 106: 795-807.

Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ. Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. Lancet 1984; i: 1091-1094.

Brudvik P, Rygh P. The initial phase of orthodontic root resorption incident to local compression of the periodontal ligament. Eur J Orthod 1993a;15:249-263.

Brudvik P, Rygh P. Non-clast cells start orthodontic root resorption in the periphery of hyalinized zones. Eur J Orthod 1993b;15: 467-480.

Brudvik P, Rygh P. Root resorption beneath the main hyalinized zone. Eur J Orthod 1994a;16: 249-263.

Brudvik P, Rygh P. Multi-nucleated cells remove the main hyalinized tissue and start resorption of adjacent root surfaces. Eur J Orthod 1994b;16(4):265-73.

Brudvik P, Rygh P. Transition and determinants of orthodontic root resorption – repair sequence. Eur J Orthod 1995a;12: 177-188.

Brudvik P, Rygh P. The repair of orthodontic root resorption: an ultrastructural study. Eur J Orthod 1995b;17: 189-198.

Burgener D, Bonjour JP, Caverzasio J. Fluoride increases tyrosine kinase activity in osteoblast-like cells: regulatory role for the stimulation of cell proliferation and Pi transport across the plasma membrane. J Bone Miner Res. 1995; 10:164-171.

Burke JC, Evans CA, Crosby TR, Mednieks MI. Expression of secretory proteins in oral fluid after orthodontic tooth movement. Am J Orthod Dentofac Orthop 2002; 121: 310-315.

Butler WT, Ritchie H. The nature and functional significance of dentine extracellular matrix proteins. Int J Dev Biol 1995; 39: 169-179.

Calvo MS, Eyre DR, Gundberg CM. Molecular basis and clinical application of biological markers of bone turnover. Endocrinol Rev 1996; 17: 333-368.

Carlson CH, Armstrong WD, Singer L. Distribution, migration and binding of whole blood fluoride evaluated with radiofluoride. Am J Physiol 1960; 199: 187-189.

.

Caverzasio J, Palmer G, Suzuki A, Bonjour J-P Mechanism of the Mitogenic Effect of Fluoride on Osteoblast-like Cells : Evidences for a G Protein-Dependent Tyrosine Phosphorylation Process. J Bone Miner Res. 1997; 12: 1975-1983.

Caverzasio J, Palmer G, Bonjour JP. Fluoride: Mode of Action. Bone 1998 ; 22 : 585-589.

Chachra D, Turner CH, Dunipace AJ, Grynpas MD. The effect of fluoride treatment on bone mineral in rabbits. Calcif Tissue Int 1999; 64: 345-351.

ChambersTJ, Fuller K. Bone cell predispose endosteal surfaces to resorption by exposure of bone mineral to osteoclastic contact. J Cell Sci 1985; 76: 155-165.

Chavassieux P, Pastoureau P, Boivin G, Charhon SA, Chapuy MC, Delmas PD, Meunier PJ. Effects of sodium fluoride on bone remodelling in ewes. J Bone Miner Res. 1988; 2 (Suppl. 1): 359.

Chavassieux P. Bone effects of fluoride in animal models in vivo. A review and recent study. J Bone Miner Res 1990; 5 suppl 1: S95-99.

Chavassieux P, Pastoureau P, Boivin G, Chapuy MC, Delmas PD, Meunier PJ. Dose effects on ewe bone remodelling of short-term sodium fluoride administration- a histomorphometric and biochemical study. Bone 1991; 12: 421-427.

Chavassieux P, Pastoureau P, Boivin G, Chapuy MC, Delmas PD, Meunier PJ. Fluoride –induced bone changes in lambs during and after exposure to sodium fluoride. Osteopor Int. 1991; 2: 26-33.

Chavassieux P, Chenu C, Valentin-Opran A, Delmas PD, Boivin G, Chapuy MC, Meunier PJ. The in vitro exposure to sodium fluoride does not modify activity or proliferation of human osteoblastic cells in primary cultures. J Bone Miner Res 1993; 8: 37-44.

Cheng PT, and Bader SM. Effects of fluoride on rat cancellous bone. Bone Miner. 1990; 11: 156-161.

Chenu C, Colucci S, Grano M, Zigrino P, Baratollo R, Zambonin G, Baldini N, Vergnaud P, Delmas PD, Zallone AZ. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signalling in human osteoblast-like cells. J Cell Biol 1994; 4: 1149-1158.

Christiansen P, Steiniche T, Brixen K, Hessov I, Melsen F, Charles P, et al. Primary hyperparathyroidism: biochemical markers and bone mineral density at multiple skeletal sites in Danish patients. Bone 1997; 21: 93-9.

Christner P. Robinson P. Clark CC. A preliminary characterization of human cementum collagen. Calcif Tiss Res 1977; 23(2):147-50.

Christoffersen J, Landis WJ. A contribution with review to the description of mineralization of bone and other calcified tissues *in vivo*. Anat Rec 1991; 230: 435-450.

Cole DE, Hanley DA. Osteocalcin. In: Hall, BK (Ed.), Bone Matrix and Bone Specific Products. CRC Press, London, UK, pp 239-294.

Cole AA, Walters LM. Tartrate-resistant acid phosphatase in bone and cartilage following decalcification and cold-embedding in plastic. J Histochem and Cytochem 1987;35: 203-206.

Colford J, Sailer D, Langman C. Five osteocalcin assays compared : tracer specificity, fragment interference and calibration. Clin Chem 1997; 43: 1240-1241.

Collins MK, Sinclair PM. The local use of vitamin D to increase the rate of orthodontic tooth movement. Am J Orthod Dentofac Orthop 1988; 94: 278-284.

Compston, J. Mechanisms of Bone Loss and Gain in Untreated and Treated Osteoporosis. Endocrine 2002; 17: 21-27.

Dandona P, Coumar A, Gill DS, Bell J, Thomas M. Sodium fluoride stimulates osteocalcin in normal subjects. Clin Endocrin 1988; 29: 437-441.

Davidovitch Z, Shanfeld JL. Cyclic AMP levels in alveolar bone of orthodontically-treated cats. Arch Oral Biol 1975;20:567-574.

Deane DD, Schwartz Z, Bonewald L, Muniz OE, Morales S, Gomez R, Brooks BP, Qiao M, Howell DS, Boyan BD. Matrix vesicles produced by osteoblast-like cells in culture become significantly enriched in proteoglycan-degrading metalloproteinases after addition of b-glycerophosphate and ascorbic acid. Calcif Tissue Int 1994; 54: 399-408.

Delmas PD, Chatelain P, Malaval L. Serum bone-gla protein in growth hormone-deficient children. J Bone Min Res. 1986; 1: 333-338.

Delmas PD, Christiansen C, Mann KG, Price PA. Bone Gla protein (osteocalcin) assay standardization report. J Bone Miner Res 1990; 5: 5-11.

Delmas PD. Clinical use of biochemical markers of bone remodelling in osteoporosis. Bone 1992; 13: S17-21.

D'Errico JA, MacNeil RL, Takata T, Berry J, Strayhorn C, Somermen MJ. Expression of bone associated markers by tooth root lining cell, in situ and in vitro. Bone 1997; 20: 117-126.

De Vries IG, Coomans D, Wisse E. Immunocytochemical localization of osteocalcin in human and bovine teeth. Calcif Tiss Int 1988; 43: 128-130.

Dixon V, Read MJF, O'Brien KD, Worthington HV, Mandall NA. A randomized clinical trial to compare three methods of orthodontic space closure. J Orthod. 2002; 29: 31-36.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C. Increased bone formation in osteocalcin deficient mice. Nature 1996; 382: 448-452.

Duda RJ, O'Brien JF, Katzmann JS, Peterson JM, Mann KG, Riggs BL. Concurrent assays of circulating bone gla-protein and bone alkaline phosphatase: effects of sex, age and metabolic bone disease. J Endocrinol Metab 1988; 66: 951-957.

Eanes ED and Reddi AH. The effect of fluoride on bone mineral apatite. Metab Bone Dis Rel Res. 1979; 2: 3-10.

Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL. Serum bone gla-protein compared to bone histomorphometry in endocrine diseases. Bone 1985; 6: 339-341.

Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL. Bone formation rate in older normal women: concurrent assessment with bone histomorphometry in endocrine diseases. Bone 1985; 6: 339-341.

Eble DM, Deaton TG, Wilson FC. Fluoride concentrations in human and rat bone. J Public Health Dent 1992; 52: 288-291.

Einhorn TA, Wakley GK, Linkhart S. Incorporation of sodium fluoride into cortical bone does not impair the mechanical properties of the appendicular skeleton in rats. Calcif Tissue Int. 1992; 51: 127-131.

Ekstrand J, Ericsson Y, Rosell S. Absence of protein-bound fluoride from human blood plasma. Arch Oral Biol 1977; 22: 229-232.

Eley BM and Cox SW. Advances in periodontal diagnosis 10. Potential markers of bone resorption. BDJ 1998; 184: 489-492.

Engström C, Granström G, Thilander B. Effect of orthodontic force on periodontal tissue metabolism. Am J Orthod Dentofac Orthop 1988; 93: 486- 495.

Eriksen EF, Vesterby A, Kassem M. Bone remodeling and bone structure. In: Mundy GR, Martin TJ, editors. Physiology and pharmacology of bone. Heidelberg: Springer-Verlag, 1993.

Faccini JM. Inhibition of bone resorption in the rabbit by fluoride. Nature 214; 1269-1271.

Farley JR, Werdegal JE, Baylink DJ. Fluoride directly stimulates proliferation and alkaline phosphatase activity in bone-forming cells. Science. 1983; 222: 330-332.

Farley JR, Tarbaux N, Hall S, Baylink DJ. Evidence that fluoride-stimulated [^{3}H]-thymidine incorporation in embryonic chick calvarial cultures is dependent on the presence of a bone cell mitogen, sensitive to changes in the phosphate concentration, and modulated by systemic skeletal effectors. Metab. 1988; 37:988-995.

Farley JR, Hall SL, Tanner MA, Wergedal JE. Specific activity of skeletal alkaline phosphatase in human osteoblast-line cells regulated by phosphate, phosphate esters, and phosphate analogues and release of alkaline phosphatase activity inversely regulated by calcium. Journal of Bone and Mineral Research 1994;9: 497-508.

Farrugia W and Melick RA. Metabolism of osteocalcin. Calc Tissue Int. 1986; 39: 234-238.

Fassbender WJ, Steinhauer B, Stracke H, Schumm-Draeger PM, Usadel KH. Validation of a new automated immunoassay for measurement of intact osteocalcin. Clin Lab. 2002; 48: 31-38.

Fedde KN. Human osteosarcoma cells spontaneously release matrix-vesicle-like structures with the capacity to mineralize. Bone and Mineral 1992;17: 145-151.

Ferrier J, Illeman A, Zakshek E. Transient and sustained effects of hormones and calcium on membrane potential in a bone cell clone. J Cell Physiol. 1985; 122: 53-58.

Franceschi RT, Romano PR, Park KY. Regulation of type I collagen synthesis by 1,25 dihydroxyvitamin D3 in human osteosarcoma cells. Journal of Biological Chemistry 1988; 263: 18938-18945.

Fritsch J, Edelman A, Balsan S. Early effects of parathyroid hormone on membrane potential of rat osteoblasts in culture: role of cAMP and Ca^{2+}. J Bone Miner Res 1988; 3: 547-554.

Garnero P, Grimaux M, Seguin P, Delmas PD. Characterization of immunoreactive forms of human osteocalcin generated in vivo and in vitro. J Bone Miner Res 1994; 9: 255-64.

Gertz BJ, Clemens JD, Holland SD, Yuan W, Greenspan S. Application of a new serum assay for type I collagen cross-linked N-telopeptides: assessment of diurnal changes in bone turnover with and without alendrolate treatment. Calcif Tiss Int 1998; 63: 102-106.

Giannobile WV, Lynch SE, Denmark RG, Paquettee DW, Fiorellini JP, Williams RC. Crevicular fluid osteocalcin and pyridinoline cross-linked carboxyterminal telopeptide of type-I collagen (ICTP) as markers of rapid turnover in periodontitis. A pilot study in beagle dogs. J Clin Periodontol 1995; 22: 901-910.

Giavaresi G, Fini M, Gnudi S, Mongiorgi R, Ripamonti C, Zati A, Giardino R. The mechanical properties of fluoride-treated bone in the ovariectomized rat. Calcif Tissue Int, 1999; 65: 237-241.

Gibson JM, King GJ, Keeling SD. Long-term orthodontic tooth movement response to short-term force in the rat. Angle orthod 1992; 62: 211-215.

Glowacki J. Studies of the regulation of bone synthesis and resorption. In: Factors and Mechanisms Influencing Bone Growth, eds. A.D. Dixon and B.G. Sarnat (AR Liss, New York) pp.83-91 (1982).

Glowacki J, Lian JB. Impaired recruitment and differentiation of osteoclast progenitors by osteocalcin-deplete bone implants. Cell Diff 1987; 21: 247-254.

Glowacki J, Rey C, Glimcher MJ, Cox KA, Lian J. A Role for Osteocalcin in Osteoclast Differentiation. J Cell Biochem 1991; 45: 292-302.

Gowen M. Cytokines and skeletal disorders. **Baxter A, Ross R**, eds. Cytokine Interactions and their Control. London: John Wiley and Sons, 125-137; 1991.

Grieve WG, Johnson GK, Moore RN, Reinhardt RA, DuBois LM. Prostaglandin E (PGE) and interleukin-1 beta levels in gingival crevicular fluid during human orthodontic movement. Am J Orthod Dentofac Orthop 1994; 105: 369-374.

Griffiths GS, Moulson AM, Petrie A, James IT. Evaluation of osteocalcin and pyridinium crosslinks of bone collagen as markers of bone turnover in gingival crevicular fluid during different stages of orthodontic treatment. J Clin Periodontol 1998; 25: 492-498.

Grynpas MD, Cheng P-T. Fluoride reduced the rate of dissolution of bone. Bone Miner 1988; 5: 1-9.
Gundberg CM, Lian JB, Gallop PM. Measurements of gamma-carboxyglutamate and circulating osteocalcin in normal children and adults. Clin Chim Acta 1983; 128: 1-8.

Gundberg CM, Wilson PS, Gallop PM, Parfitt AM. Determination of osteocalcin in human serum: results with two kits compared with those by a well-characterized assay. Clin Chem 1985; 31: 1720-1723.

Hall BK. Earliest evidence of cartilage and bone development in embryonic life. Clin Orthop Rel Res; 1987; 225: 255.

Hall BK. Embryonic Bone Formation with Special Reference to Epithelial-Mesenchymal Interactions and Growth Factors. In: Bone, Vol 8: Mechanisms of Bone Development and Growth. Hall BK, editor. CRC Press, Inc. Florida. p165-166. 1994.

Hannon R, Eastell R. Biochemical markers of bone turnover. In: Tovey FI, Stamp TCB, eds. The Measurement of Metabolic Bone Disease. London: Parthenon Publishing Group, 1995: 21-47.

Hannon R, Blumsohn A, Naylor K, Eastell R. Response of biochemical markers of bone turnover to hormone replacement therapy: impact of biological variability. J Bone Miner Res 1998; 13: 1124-1133.

Harris H. The human alkaline phosphatase, what we know and what we don't know. Clinica Chimica Acta 1989; 186: 133-136.

Harris SA, Tau KR, Turner RT, Spelsberg TC. Estrogens and progestins. In : Principles of bone biology. Bilezikian JP, Raisz LG, Rodan GA, Eds. San Diego: Academic Press, pp 507-520.

Harter LV, Hruska KA, Duncan RL. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. Endocrinology 1995; 136: 528-535.

Haruyama N, Igarashi K, Saeki S, Otsuka-Isoya M, Shinoda H, Mitani H. Estrous-cycle-dependent Variation in Orthodontic Tooth Movement. J Dent Res 2002; 81: 406-410.

Hashimoto F, Kobayashi Y, Matakai S, Kobayashi K, Kato Y, Sakai H. Administration of osteocalcin accelerates orthodontic tooth movement induced by a closed coil spring in rats. Eur J Orthod 2001; 23: 535-545.

Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. Journal of Biological Chemistry 261: 12665-12674; 1986.

Hauschka PV. Calcium-dependent alpha-helical structure in osteocalcin. Biochemistry. 1986; 16: 258-272.

Hauschka PV, Lian JB, Cole C, Gundberg CM, Osteocalcin and MGP: vitamin K-dependent proteins in bone. Physiol Rv 1989; 69: 990-1047.

Häuselmann HJ and Rizzoli, R. A comprehensive review of treatments for postmenopausal osteoporosis. Osteoporos Int 2003; 14:2-12.

Hellsing E and Hammarstrøm L. The effects of pregnancy and fluoride on orthodontic tooth movement in rats. Eur J Orthod. 1991; 13: 223-230.

Hellsing E and Hammarstrøm L. The hyaline zone and associated root surface changes in experimental orthodontics in rats: a light and scanning electron microscope study. Eur J Orthod 1996; 18: 11-18

Heymann D, Guicheux J, Gouin F, Passuti N, Daculsi G. Cytokines, Gowth Facors and Osteoclasts. Cytokine 1998; 10: 155-168.

Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology 1997; 138: 3849-3858.

Holtrop ME. Light and electron microscopic structure of bone-forming cells. Hall BK, ed. Bone: Volume I. The Osteocyte. New Jersey: The Telford Press, 1-39; 1990.

Horton M. Bone Cell Genes, molecular Analysis. In: Encyclopedia of Human Biology, Vol. 1 (R. Dulbecco, Ed), Academic Press, San Diego, pp 761-770.

Horton M and Davies J. Perspectives: adhesion receptors in bone. J Bone Miner Res 1989; 4: 803-808.

Hughes B, King GJ. Effect of orthodontic appliance reactivation during the period of peak expansion in the osteoclast population. Anat Rec 1998; 251: 80-86.

Igarashi K, Miyoshi K, Shinoda H, Saeki S, Mitani H. Diurnal variation in tooth movement in response to orthodontic force in rats. Am J Orthod Dentofacial Orthop 1998; 114: 8-14.

Ingram, RT, Park YK, Clarke BL, Fitzpatrick LA. Age- and Gender-related Changes in the Distribution of Osteocalcin in the Extracellular Matrix of Normal Male and Female Bone. Possible involvement of Osteocalcin in Bone Remodelling. J Clin Invest 1994; 93: 989-997.

Mah J, Prasad N. Dentine phosphoproteins in gingival crevicular fluid during root resorption. Eur J Orthod. 2004; 26: 25-30.

Insoft M, King GJ, Keeling SD. The measurement of acid and alkaline phosphatase in gingival crevicular fluid during orthodontic tooth movement. Am J Orthod Dentofac Orthop. 1996; 109: 287-296.

Ishiguro K, Nakagaki H, Takeuchi K, Mukai M, Yoshioka I, Miyauchi K, Robinson C, Weatherell JA. Distribution of fluoride in the dental tissues and the supporting mandibular bone from the same individual. Arch Oral Biol. 1994; 39: 535-537.

Ishizuka Y, Tanne K. Masticatory muscle activity in rats during the day. Dent Japan 1995; 32: 83-86.

Ito T, Nakagaki H, Kato K, Kondo K, Isogai A, Adachi K, Negoro M, Huang AB, Nguyen TT, Robinson C. The cessation of fluoridated water administration and the fluoride distribution profiles in rat molar cementum. Caries Res 1997; 31: 390-396.

Ivanovski S, Li H, Haase HR, Bartold PM. Expression of bone associated macromolecules by gingival and periodontal ligament fibroblasts. J Periodont Res 2001; 36: 131-141.

Ivaska KK, Hellman J, Likojarvi J, Kakonen S-M, Gerdhem P, Akesson K, Obrant KJ, Pettersson K, Vaananen HK. Identification of novel proteolytic forms of osteocalcin in human urine. Biochem Biophys Res Comm. 2003; 306: 973-980.

Jones S, Boyde A. The resorption of dentine and cementum. In: Davidovitch Z, ed. Biological mechanisms of tooth eruption and root resorption. 1988: 293-302, 335 – 54.

Jowsey J, Riggs BL, Kelly PJ, Hoffman DL. Effects of combined therapy with sodium fluoride, vitamin D and calcium in osteoporosis. Am J Med. 1972; 53: 43-49.

Kagayama M, Li HC, Zhu J, Sasano Y, Hatakeyama Y, Mizoguchi I. Expression of osteocalcin in cementoblasts forming acellular cementum . J Perio Res 1997; 32: 273-278.

Kanbur NO, Derman O, Sen TA, Kinik E. Osteocalcin. A biochemical marker of bone turnover during puberty. Int J Adolesc Med Health. 14:235-44; 2002.

Kassem M, Mosekilde L, Eriksen EF. Effects of fluoride on human bone cells in vitro: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. Eur J Endocrinol 1994; 130: 381-386.

Kato K, Nakagaki H, Robinson C, Weatherell JA. Distribution of fluoride across cementum, dentine and alveolar bone in rats. Caries Res. 1990; 24: 117-120.

Kato S, Nakagaki H, Toyama, Kanayama T, Arai M, Matsumoto S, Strong M, Robinson C. Fluoride profiles in the cementum and root dentine of human permanent anterior teeth extracted from adult residents in a naturally fluoridated and a non-fluoridated area. Gerodontol 1997; 14: 1-8.

Khokher MA, Dandona P. Fluoride stimulates [^{3}H]-thymidine incorporation and alkaline phosphatase production by human osteoblasts. Metabolism 1990; 39:1118-1121. line:cAMP dependent and cAMP-independent calcium channels. J Biol Chem 1987; 262: 7711-7718.

King GJ, Fischlschweiger W. the effect of force magnitude on extractable bone resorptive activity and cemental cratering in orthodontic tooth movement. J Dent Res 1982; 61: 775-779.

King GJ, Keeling SD, Wronski TJ. Histomorphometric study of alveolar bone turnover in orthodontic tooth movement. Bone 1991;12: 401-409.

King GJ, Keeling SD. Orthodontic bone remodelling in relation to appliance decay. Angle Orthod 1995; 2: 129-140.

King GJ, Latta L, Rutenberg J, Ossi A, Keeling A. Effect of appliance removal on alveolar bone turnover in rats. J Dent Res 1995; 74: 927 (Abstr).

King GJ, Latta L, Rutenberg J, Ossi A, Keeling SD. Alveolar bone turnover and tooth movement in male rats after removal of orthodontic appliances. Am J Orhod Dentofac Orthop 1997; 111: 266-275.

Kirker-Head CA, Gerhart T, Henning G, et al. Long-term healing of large mid-femoral segmental defects in sheep using recombinant human bone morphogenetic protein-2 and inactive decalcified ovine bone matrix. Trans 40th Ann Orthopaed Res Soc 1994; 19: 505.

Klaushofer K, Peterlik M. Pathophysiology of osteoporosis. In : Bröll H, Dambacher MA, editors. Osteoporosis: a guide to Diagnosis and treatment. Rheumatology. Vol 18: Switzerland: Basel, Karger pp12-27.

Kleerekoper M. Fluoride and the Skeleton. Crit Rev Clin Lab Sci. 1996; 33: 139-161

Kobayashi Y, Takagi H, Sakai H, Hashimoto F, Mataki S, Kobayashi K, Kato Y. Effects of local administration of osteocalcin on experimental tooth movement. Angle Orthod 1998; 68: 259-266.

Konoo T. Experimental study on the effect of the cheeks and tongue on the teeth and the periodontal tissues. (Tooth elongation by elimination of the cheek and tongue pressures). J Kyushu Dent Soc 1988; 42: 48-64.

Kopp JB Robey PG. Sodium fluoride does not increase human bone cell proliferation or protein synthesis in vitro. Calcif Tiss Int. 1990; 47: 221-229.

Kragstrup J, Richards A, Fejerskov O. Experimental osteofluorosis in the domestic pig: a histomorphometric study of vertebral trabecular bone. J Dent Res 1984; 63: 885-889.

Kunimatsu K, Mataki S, Tanaka H. A cross-sectional study of osteocalcin levels in gingival crevicular fluid from periodontitis patients. J Periodontol 1993; 64: 865-869.

Lau KH, Farley JR, Freeman TK, Baylink DJ. A proposed mechanism of the mitogenic action of fluoride on bone cells: inhibition of the activity of an osteoblastic acid phosphatase. Metabolism 1989; 38: 858-68.

Lau KH, Baylink DJ. Molecular mechanism of action of fluoride on bone cells. J Bone Miner Res. 1998; 13: 1660-1667.

Lau KH and Baylink DJ. Osteoblastic tartrate-resistant acid phosphatase: its potential role in the molecular mechanism of osteogenic action of fluoride. J Bone Miner Res. 2003; 18: 1897-1900.

Lakatos P, Blumsohn A, Eastell R, Tarjan G, Shinoda H, Stern PH. Circadian rhythm of in vitro bone-resorbing activity in human serum. J Clin Endocrinol Metab 1995; 80: 3185-3190.

Leboy PS, Beresford JN, Devlin C, Owen ME. Dexamethasone induction of osteoblast mRNA in rat marrow stromal cell cultures. J Cell Physiol 1991; 146: 370-378.

Lee AJ, Hodges S, Eastell R. Measurement of osteocalcin. Ann Clin Biochem 2000; 37: 432-446.

Lee S and Hanson DB. Effect of fluoride dosage on bone density, sonic velocity, and longitudinal modulus of rabbit femurs. Calcif Tissue Int. 1992; 50: 88-92.

Leung KS, Fung KP, Sher AHL, Li CK, Lee KM. Plasma specific alkaline phosphatase as an indicator of osteoblastic activity. J Bone Joint Surg 1993; (Br) 75-B: 288-292.

Li R, Den Besten PK. Expression of bone protein mRNA at physiological fluoride concentrations in rat osteoblast culture. Bone Miner 1993; 22: 187-96.

Lian JB, Tassinari M, Glowacki J. Resorption of implanted bone prepared from normal and warfarin treated rats. J Clin Invest. 1984; 73: 1223-1226.

Liggett WH, Lian JB, Greenberger JS, Glowacki J. Osteocalcin promotes differentiation of osteoclast progenitors from murine long-term bone marrow cultures. J Cell Biochem 1994; 55: 190-199.

Lindskog S, Hammarstrøm L. Evidence in favour of an anti-invasion factor in cementum or periodontal membrane of human teeth. Scand J Dent Res 1980; 88: 161-163.

Lindskog S, Flores ME, Lilja E, Hammarstrøm L. Effect of a high dose of fluoride on resorbing osteoclasts *in vivo*. Scand J Dent Res. 1989; 97: 483-487.

Liu YC, Evans CA, Narayanan AS, George A. Immunodetection of dentine and cementum proteins in crevicular fluid. J Dent Res. 2000; 79: 613 (Abstract).

Lu L-H, Lee K, Imoto S, Kyomen S, Tanne K. Histological and histochemical quantification of root resorption incident to the application of intrusive force to rat molars. Eur J Orthod 1999; 21: 57-63.

Lundy MW, Farley JR, Baylink DJ. Characterization of a rapidly responding animal for fluoride-stimulated bone formation. Bone 1986; 7: 289-93.

McCauley LK and Nohutcu RM. Mediators of Periodontal Osseous Destruction and Remodeling: Principles and Implications for Diagnosis and Therapy. J Periodontol 2002; 73: 1377-1391.

Mc Culloch CAG, Nemeth E, Lowenberg , Melcher AH. Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell populations. Anat Record 1987; 219: 233-242.

McKee MD, Farach-Carson M, Butler W, Hauschka P, Nanci A
Ultrastructural immunolocalization of non-collagenous (osteopontin and osteocalcin) plasma (albumin) proteins in rat bone. J Bone Min Res (1993); 8: 485-496.

MacNeil RL, Berry J, D'Errico J, Strayhorn C, Piotrowski B, Somerman MJ. Role of two mineral-associated adhesion molecules, osteopontin and bone sialoprotein, during cementogenesis. Connect Tissue Res 1995; 33: 1-7.

MacNeil RL, D'Errico JA, Ouyang H, Berry J, Strayhorn C, Somerman MJ. Isolation of murine cementoblasts: unique cells or uniquely-positioned osteoblasts. Eur J Oral Sci 1998; 106 Suppl 1: 350-356.

McSheehy PMJ, Chambers TJ. Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption. Endocrinology 1986; 119: 1654-1659.

Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn RJ. Recruitment of osteoclast precursors by purified bone matrix constituents. J Cell Biol. 1982; 92: 227-238.

Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodeling. New Engl J Med. 1995;322: 305-311.

Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. New Engl J Med 2000; 332: 305-311.

Marie PJ, De Vernejoul MC and Lomri A. Stimulation of bone formation in osteoporosis patients treated with fluoride associated with increased DNA synthesis by osteoblastic cells in vitro. J Bone Min Res 1992; 7: 103-113.

Markowitz ME, Gundberg CM, Rosen JF. The circadian rhythm of serum osteocalcin concentrations: effects of 1,25 dihydroxyvitamin D administration. Calcif Tissue Int 1987; 40: 179-183.

Matsuda N, Yokoyama K, Takeshita S, Watanabe M. Role of epidermal growth factor and its receptor in mechanical stress-induced differentiation of human periodontal ligament cells in vitro. Arch Oral Biol 1998; 43: 987-997.

Meikle MC, McGarrity AM, Thomson BM, Reynolds JJ. Bone-derived growth factors modulate collagenase and TIMP activity and Type I collagen degradation by mouse calvarial osteoblasts. Bone and Min 1991; 21: 41-55.

Meikle MC. Craniofacial development, growth and evolution. Bateson Publishing Norfolk, 2002.

Melcher AH, Cheong T, Cox J, Nemeth E. Synthesis of cementum-like tissue in vitro by cells cultured from bone. J Periodont Res 1986; 21: 592-612.

Melsen B, Falkenberg Topp L, Melsen HM, Terp S. Force systems developed from closed coil springs. Eur J Orthod. 1994; 16: 531-539.

Melsen F, Eriksen EF, Mosekilde L. Clinical Aspects of Fluoride in Bone. In: Fluoride in Dentistry 2nd edition. Fejers O, Ekstrand J, Burt BA, Eds. Munksgaard 1996.

Melsen B. Biological reaction of alveolar bone to orthodontic tooth movement. Angle Orthod 1999; 69: 151-158.

Merle B, Delmas PD. Normal carboxylation of circulating osteocalcin (bone gla-protein) in Paget's disease of bone. Bone Miner. 1990; 11: 237-245.

Metzger Z, Weinstock B, Dotan M, Narayanan AS, Pitaru S. Differential chemotactic effect of cementum attachment protein on periodontal cells. J Periodontol Res 1998; 33: 126-129.

Meunier PJ. Fluoride therapy for vertebral osteoporosis. In: Munro H, Schlierf D, editors. Nutrition of the elderly. Nestlé Nutrition Workshop Series Vol 29. 1992; Nestlé Ltd. New York: Vevey/ Raven Press Ltd. pp177-185.

Miyajima K, Ohno Y, Iwata T, Tanida K, Izuka T. The lactic acid and citric acid content in the gingival fluid of orthodontic patients. Aichi Gakuin Dental Sci. 1991; 4: 75-82.

Mohan S, Baylink DJ. Bone growth factors. Clinical Orthopaedics. 262:30-48;1990.

Mundy G. Peptides and growth regulatory factors in bone. Rheum Dis Clin N Am 1994; 20: 577-588.

Muyzer G, Sandberg P, Knapen MHJ, Vermeer C, Collins MJ, Westbroek P. Preservation of bone protein osteocalcin in dinosaurs. Geology 1992; 20: 871-874.

Nakagaki H, Weatherell JA, Strong M, Robinson C. Distribution of fluoride in human cementum. Archs Oral Biol 1985; 30: 101-104.

Nakagaki H, Kawai K, Murakami T, Sakakibara Y, Ohno N, Weatherell JA, Robinson C. Fluoride distribution and histological structure of human cementum. Arch Oral Biol. 1988; 33: 257-264.

Nakane S, Kameyama Y. Root resorption caused by mechanical injury of the periodontal soft tissues in rats. J Periodont Res 1987; 22: 390-395.

Nakashima K, Roehrich N, Cimasoni G. Osteocalcin, prostaglandin E_2 and alkaline phosphatase in gingival crevicular fluid: their relations to periodontal status. J Clin Periodontol 1994; 21: 327-333.

Nicholson G, Bryant AE, Macdonald IA, Hall GM. Osteocalcin and the hormonal, inflammatory and metabolic response to major orthopaedic surgery. Anaesthesia 2002; 57: 319-325.

Nielsen HK, Brixen K, Mosekilde L. Diurnal rhythm and 24-hour integrated concentrations of serum osteocalcin in normals: influence of age, sex, season and smoking habits. Calcif Tissue Int.1990; 47: 284-290.

Nielson HM, Andreassen TT, Ledet T, Oxlund H. Local injection of TGF-ß increases the strength of tibial fractures in the rat. Acta Orthopaed Scand 1994; 65: 37-41.

Noonan K, Kalu ME, Holownia P, Burrin JM. Effect of different storage temperatures, sample collection procedures and immunoassay methods on osteocalcin measurements. Eur J Clin Chem Clin Biochem 1996; 34: 841-4.

O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CA, Stewart SA, Manolagas SC, Weinstein RS. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinol 2004; 145: 1835-1841.

Oguro A, Kawase T, Orikasa M. NaF induces early differentiation of murine bone marrow cells along the granulocytic pathway but not the monocytic or preosteoclastic pathway in vitro. Soc In Vitr Biol J 2003; 39: 243-248.

Ohta T, Werdegal JE, Matsuyama T, Baylink DJ, Lau KH. Phenytoin and fluoride act in concert to stimulate bone formation and to increase bone volume in rats. Calcif Tiss Int 1995; 56: 390-397.

Okuda A, Kanehisa J, Heersche JNM. The effects of sodium fluoride on the resorptive activity of isolated osteoclasts. J Bone Miner Res 1990 (suppl 1): S115-S120.

Oudet C & Petrovic A. Growth rhythms of the cartilage of the mandibular condyle: effects of orthopaedic appliances. Int J Chronobiol 1978; 5 : 545-564.

Pak CY, Sakhaee K, Zerwekh JE, Parcel C, Peterson R, Johnson K. Safe and edffective treatment of osteoporosis with intermittent slow release sodium fluoride: augmentation of vertebral bone mass and inhibition of fractures. J Endocrin Metab 1989; 68: 150-9.

Pak CYC, Zerwekh JE, Antich P. Anabolic effects of fluoride on bone. Trends Endocrinol Metab 1995; 6: 229-234.

Papagerakis P, Berdal A, Mesbah M, Peuchmaur M, Malaval L, Nydegger J, Simmer J, Macdougall M. Investigation of Osteocalcin, Osteonectin, and Dentine sialophosphoprotein in Developing Human Teeth. Bone 2002; 30: 377-385.

Pavlin D, Zadro R, Gluhak-Heinrich J. Sequence of stimulation of osteoblast-associated genes in vivo during mechanically-induced osteogenesis: parallel stimulation of osteocalcin and type- I collagen. Connect Tissue Res 2001; 42: 345-351.

Pavlin D, Gluhak-Heinrich J. Effect of loading on periodontal cells. Crit Rev Oral Biol Med 2001; 12: 414-424.

Posner AS, Eanes ED, Harper RA, Zipkin I. X-Ray diffraction analysis of the effect of fluoride on human bone apatite. Arch Oral Biol. 1963; 8: 549-570.

Posner AS. The Effect of Fluoride on Bone Mineralization. In: Clinical Aspects of Fluoride in Bone. In: Fluoride in Dentistry 2nd edition. Fejers O, Ekstrand J, Burt BA, Eds. Munksgaard 1996 pp 88-89.

Power MJ and Fottrell PF. Osteocalcin: Diagnostic methods and clinical applications. Crit Rev Clin Lab Sci. 1991; 28: 287-335.

Power MJ, O'Dwyer B, Breen E, Fottrell PF. Osteocalcin concentrations in plasma prepared with different anticoagulants. Clin Chem 1991; 37: 281-284.

Price PA, Baukol SA. 1,25 $(OH)_2$ vitamin D_3 increases synthesis of the vitamin K dependent protein by osteosarcoma cells. J Biol Chem 1980; 256: 11660-11663.

Price PA, Williamson MK. Effect of warfarin on bone: studies on the vitamin-K-dependent protein of rat bone. J Biol Chem 1981; 256:12754-12759.

Price RI, Williamson MK, Lothringer JW. Origin of the vitamin K-dependent protein found in plasma and its clearance by kidney and bone. J Biol Chem 1981; 256: 12760-12766.

Price PA, Williamson MK, Lothringer JW. Origin of the vitamin-K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem. 1981; 256: 12760-12766.

Price PA. Gla-containing proteins of bone. Connect Tissue Res 1989; 21: 51-60.

Proffit WR. Contemporary Orthodontics. St Louis, Calif: Mosby-Year Book Inc, 1999; 296–325.

Rao HV Beliles RP Whitford GM Turner CH. A Physiologically Based Pharmacokinetic Model for Fluoride Uptake by Bone. Reg Tox Pharm 22: 30-42; 1995.

Ream LJ. The effects of short-term fluoride ingestion on bone formation and resorption in the rat femur. Cell Tissue Res. 1981; 221: 421-430.

Ream LJ. The effects of short-term fluoride ingestion on bone formation and resorption in the rat femur. Cell Tissue Res. 1981; 221: 421-430.

Reed BY, Zerwekh JE, Antich PP, Pak CYC. Fluoride-stimulated [^{3}H]thymidine uptake in a human osteoblastic osteosarcoma cell line is dependent on transforming growth factor beta. J Bone Miner Res. 1993; 8: 19-25.

Reid IR, Chapman TR, Fraser AD, Davies AS, Surus J, Meyer J. Low serum osteocalcin levels in glucocorticoid treated asthmatics. J Clin Endocrinol Metab 1986; 62: 379-383.

Reinholt FK, Hultenby A, Oldberg A, Heinegard D. Osteopontin: a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 1990; 87: 4473-4475.

Reitan K. The initial tissue reaction incident to tooth movement as related to the influence of function. Acta Odontol Scand 1951;(suppl 10):1-60.

Reitan K. Tissue behaviour during orthodontic tooth movement. Am J Orthod 1960; 46: 881-900.

Reitan K. Biomechanical principles and reactions. In: Orthodontics Current Principles and Techniques. Graber TM, Swain BF (eds) St Louis: CV Mosby; 1985. p.101-192.

Riggs BL, Hodgson SF, Eastell R. The Role of Sodium Fluoride in the Treatment of Osteoporosis. In: Clinical Disorders of bone and Mineral Metabolism. Eds Kleerekoper M and Krane SM. Mary Ann Liebert, Inc Publishers NY 1989.

Ringe JD, Rovati LC. Treatment of osteoporosis in men with fluoride alone or in combination with bisphosphonates. Calcif Tissue Int 2001; 69: 252-255.

Risteli L, Risteli J. Biochemical markers of bone metabolism. Annals of Medicine 1993; 25: 385-393.

Ritz S, Turzynski A, Schutz HW, Hollmann A, Rochholz G. Identification of osteocalcin as a permanent aging constituent of the bone matrix: basis for an accurate age at death determination. Forensic Sci Int 12: 13-26; 1996.

Roach HI. Why does bone matrix contain non-collagenous proteins? The possible roles of osteonectin, osteopontin and bone sialoprotein in bone mineralization and resorption. Cell Biol Int. 1994; 6: 617-628.

Roberts WE, Chase DC. Kinetics of cell proliferation and migration associated with orthodontically induced osteogenesis. J Dent Res 1981; 60: 174-181.

Roberts WE, Klingler E, Mozsary PG. Circadian rhythm of mechanically mediated differentiation of osteoblasts. Calcif Tissue Int 1984; 36: s62-s66.

Roberts WE, Huja S, Roberts JA. Bone modeling: biomechanics, molecular mechanisms, and clinical perspectives. Semin Orthod 2004;10: 123-161

Robey PG. Vertebrate mineralized matrix proteins: structure and function. Conn Tiss Res 1996; 35: 185-190.

Robey PG and Boskey AL. (1996) "The Biochemistry of Bone." Osteoporosis, R Marcus, D Feldman, and J Kelsey, eds, Academic Press, New York, 241-263.

Robins SP. Biochemical markers for assessing skeletal growth. Eur J Clin Nutr 1994; 48 Suppl 1: S199-S209.

Rodan GA, Rodan SB. Expression of the osteoblast phenotype. Peck WA, ed. Bone Mineral Research. Vol. 2. Amsterdam : Elsevier, pp 244-262; 1983.

Rodan GA. Introduction to bone biology. Bone 1992;13: s3-6

Rodan GA, Martin TJ. Role of osteoblasts in hormonal control of bone resorption- a hypothesis. Calcif Tissue Int. 1981; 33: 349-351.

Rodriguez JP and Rosselot G. Sodium Fluoride Induces Changes on Proteoglycans Synthesized by Avian Osteoblasts in Culture. J Cell Biochem 2001; 83:607-616.

Rody WJ, King GJ, Gu G. Osteoclast recruitment to sites of compression in orthodontic tooth movement. Am J Dentofac Orthop 2001; 120: 477-489.

Roodman GD. Advances in bone biology: the osteoclast. Endocrine Reviews 1996; 17: 308-332.

Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. Clin Chem 1995; 41: 1439-1445.

Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. Clin Chem 1995; 41: 1439-1445.

Rubin CT, McLeod KJ. Promotion of bony ingrowth by frequency-specific, low-amplitude mechanical strain. Clin Orthop 1994; 298: 165-174.

Rygh P. Periodontal response to tooth-moving force: is trauma necessary? In: Orthodontics-state of the art. 1986; Graber LW, Ed. St Louis: Mosby, pp. 100-115.

Samuels RHA, Rudge SJ, Mair LH. A comparison of the rate of space closure using a nickel-titanium spring and an elastic module: a clinical study. Am J Orthod Dentofac Orthop 1993; 103: 464-467.

Samuels RHA, Rudge SJ, Mair LH. A clinical study of space closure with nickel-titanium springs and an elastic module. Am J Orthod Dentofac Orthop 1998; 114: 73-79.

Schnitzler CM, Wing JR, Gear KA, Robson HJ. Bone fragility of the peripheral skeleton during fluoride therapy for osteoporosis. Clin Orthop Rel Res. 1990; 261: 268-275.

Scott JH, Symons NBB. Introduction to Dental Anatomy. Ninth Ed. Churchill Livingstone Press 1982: pp 263-272.

Shinoda H, Stern PH. Diurnal rhythms in Ca transfer into bone, Ca release from bone, and bone resorbing activity in the serum of rats. Am J Physiol 1992; 262: R235-240.

Simmons DJ, Menton DN, Russell JE, Smith R, Walker WV. Bone cell populations and histomorphometric correlates to function. Anat Rec 1988; 222: 228-236.

Simmons DJ. Circadian aspects of bone biology. In: Hall BK, ed. Bone (Bone Growth-A). Boca Raton: CRC Press; 1992. pp 91-128.

Singer J, Furstman L, Bernick S. A histologic study of the effect of fluoride on tooth movement in the rat. Am J Orthod 1967; 53: 296-308.

Skojdt M, Russell G. Bone cell biology and the regulation of bone turnover. Gowen M, ed. Cytokines and Bone Metabolism. Florida: CRC Press, 1-70; 1992.

Snow GR, Anderson C. Short-term chronic fluoride administration and trabecular bone remodelling in beagles: a pilot study. Calcif Tiss Int 1986; 38: 217-221.

Sodek J, Overall CM. Matrix degradation in hard and soft connective tissues. In: Biological mechanisms of tooth eruption and root resorption. Davidovitch Z, Ed. 1988:303-12.

Sogaard CH, Mosekilde L, Schwartz W, Leidig G, Minne HW, Ziegler R. Effects of fluoride on rat vertebral body biomechanical competence and bone mass. Bone 1995; 16:163-169.

Srivastava RN, Gill DS, Moudgil A, Menon RK, Thomas M, Dandona P. Normal ionized calcium, parathyroid hypersecretion, and elevated osteocalcin in a family with fluorosis. Metabolism 1989; 38: 120-124.

Srivastava AK, Bhattacharyya, Castillo G, Mohan S, Baylink DJ. Development and application of a serum C-telopeptide and osteocalcin assay to measure bone turnover in an ovariectomized rat model. Calcif Tissue Int 2000; 66: 435-442.

Steigman S, Michaeli Y. Experimental intrusion of rat incisors with continuous loads of varying magnitude. Am J Orthod. 1981; 80: 429–436.

Steiner GG, Pearson JK, Ainamo J. Changes of the marginal periodontium as a result of labial tooth movement in monkeys. J Periodontol. 1981; 52:314–320.

Stepensky D, Kleinberg L, Hoffman A. Bone as an effect compartment: Models for uptake and release of drugs. Clin Pharmacokinet 2003; 42: 863-881.

Stutzman JJ, Petrovic AG. Bone cell histogenesis: the skeletoblast as a stem cell for preosteoblasts and for secondary-type prechondroblasts. Dixon AD, Sarnat BG, eds. Factors and Mechanisms Influencing Bone Growth. New York, NY: Alan R Liss Inc., 29-45; 1982.

Stutzmann J, Petrovic A. Human alveolar bone turn-over rate: a quantitative study of spontaneous and therapeutically induced variations. In: McNamara JA Jr, Ribbens KA, eds. Malocclusion and periodontium, Craniofacial growth series. Ann Arbor: Centre for Human Growth and development, University of Michigan; 1984. p.185-212.

Sugihara N, Hakagaki H, Kunisaki H, Ito F, Noguchi T, Weatherell JA, Robinson C. Distribution of fluoride in sound and periodontally diseased human cementum. Arch Oral Biol 1991; 36: 383-387.

Swaminatham R. Biochemical markers of bone turnover. Clinic Chim Acta 2001; 313: 95-105.

Tanaka H, Kuwada M, Shiraki M, Katayama Y. An enzyme immunoassay for osteocalcin. J Immunol Methods 1986; 94: 19-24.

Tanaka T, Morioka T, Ayasaka N, Iijima T, Kondo T. Endocytosis in odontoclasts and osteoclasts using microperoxidase as a tracer. J Dent Res 1990;69:883-889.

Takahashi N, Yamana H, Yoshiki S. Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinol 1988; 122: 1373-1382.

Taylor ML, Boyde A, Jones SJ. The effect of fluoride on the patterns of adherence of osteoclasts cultured on and resorbing dentine. Anat Embryol 1989; 180: 427-435.

Tenenbaum HC. Cellular origins and theories of differentiation. Hall BK, ed. Bone: Volume I. The Osteocyte. New Jersey: The Telford Press, 41-69; 1990.

Tenorio D, Cruchley A, Hughes FJ. Immunocytochemical investigation of the rat cementoblast phenotype. J Periodont Res 1993; 28: 411-419

Teti A, Grano M, Colucci S, Cantatore FP, Loperfido MC. Osteoblast-osteoclast relationships in bone resorption. Biochem Biophy Res Commun. 1991; 179: 634-640.

Thomsen K, Eriksen EF, Jorgensen JC, Charles P, Mosekilde L. Seasonal variation of serum bone gla- protein. Scand J Clin Lab Invest 1989; 49:605-611.

Tracy RP, Andrianorivo A, Riggs BL, Mann KG. Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. J Bone Miner Res 1990; 5: 451-461.

Tronstad L. Root resorption – a multidisciplinary problem in dentistry. In: Davidovitch Z, ed. Biological mechanisms of tooth eruption and root resorption. Columbus, Ohio;1988 p293-302.

Turner CH, Hasegawa K, Zhang W, Wilson M, Li Y, Dunipace AJ. Fluoride reduces bone strength in older rats. J Dent Res. 1995; 74: 1475-1481.

Turner CH, Hinckley WR, Wilson ME, Zhang W, Dunipace AJ. Combined effects of diets with reduced calcium and phosphate and increased fluoride intake on vertebral bone strength and histology in rats. Calcif Tiss Int 2001; 69: 51-57.

Turner RT, Francis R, Brown D, Garand J, Hannon KS, Bell NH. The effects of fluoride on bone and histomorphometry in growing rats. J Bone Miner Res. 1989; 4: 477-484.

Uematsu S, Mogi M, Deguchi T. Interleukin (IL)-1β, IL-6, tumour necrosis factor-α, epidermal growth factor, and β_2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic movement. J Dent Res. 1996a; 75: 562-567.

Uematsu S, Mogi M, Deguchi T. Increase of TGF- β_1 in gingival crevicular fluid during human orthodontic tooth movement. Arch Oral Biol 1996b; 41: 1091-1095.

Urist MR, Delange RJ, Finerman GAM. Bone cell differentiation and growth factors. Science 1983; 220: 680-686.

Van Daele PLA, Birkenhaeger JC, Pols HAP. Biochemical markers of bone turnover: an update. Nether J Med 44: 65-72; 1994.

Vignery A, Baron R. Dynamic histomorphometry of alveolar bone remodeling in the adult rat. Anat Rec 1980; 196: 191-200.

Von Fraunhofer JA, Bonds PW, Johnson BE. Force generation by orthodontic coil springs. Angle Orthod 1993; 63: 145-148.

Waddington RJ, Embery G, Samuels RH. Characterization of proteoglycan metabolites in human gingival crevicular fluid during orthodontic tooth movement. Arch Oral Biol 1994; 39: 361-368.

Waddington RJ, Langley MS. Structural analysis of proteoglycans synthesized by mineralizing bone cells in vitro in the presence of fluoride. Matrix Biol 1998; 17: 255-268.

Waddington RJ, Embery G. Proteoglycans and Orthodontic tooth movement. J Orthod. 2001; 28: 281-290.

Weaver CM. Quantification of biochemical markers of bone turnover by kinetic measures of bone formation and resorption in young healthy females. J Bone Miner Res. 1997; 12: 1714-20.

Whitford GM. Fluoride in dental practice: safety considerations. J Dent Res 1987; 66: 1056-60.

Whitford GM. The physiological and toxicological characteristics of fluoride. J Dent Res 1990; 69: 539-549.

Whitford GM. Intake and metabolism of fluoride. Adv Dent Res 1994; 8: 5-14.

Whitford GM. The Metabolism and Toxicity of Fluoride in: Monographs in Oral Science Vol.16. Ed: Myers HM, 1996. Karger Publishers.

Wilson AN, Schmid MJ, Reinhardt RA. Bone turnover markers in serum and periodontal microenvironments. J Peri Res. 2003; 38: 355-361.

Wolf G. Function of the bone protein osteocalcin: definitive evidence. Nutr Rev 1996; 54: 332-333.

Wolinsky I, Simkin A, Guggenheim K. Effects of fluoride on metabolism and mechanical properties of rat bone. Am J Physiol. 1972; 223: 46-50.

World Health Organization. World Environmental Health Criteria 36: Fluorine and Fluorides. Publisher: World Health Organization, Geneva; 1984

Yamaguchi DT, Hahn TJ, Ida-Klein A, Kleeman CR, Muallem S. Parathyroid hormone-activated calcium channels in an osteoblast-like clonal osteosarcoma cell. J Biol Chem 1987; 262: 7711-7718.

Yamasaki K, Miura F, Suda T. Prostaglandins as mediators of bone resorption induced by experimental tooth movement in rats. J Dent Res 1980; 59: 1635-1642.

Yardeni J, Gedalia I, Kohn M. Fluoride concentration of dental calculus, surface enamel and cementum. Archs Oral Biol 1963;8: 697-701.

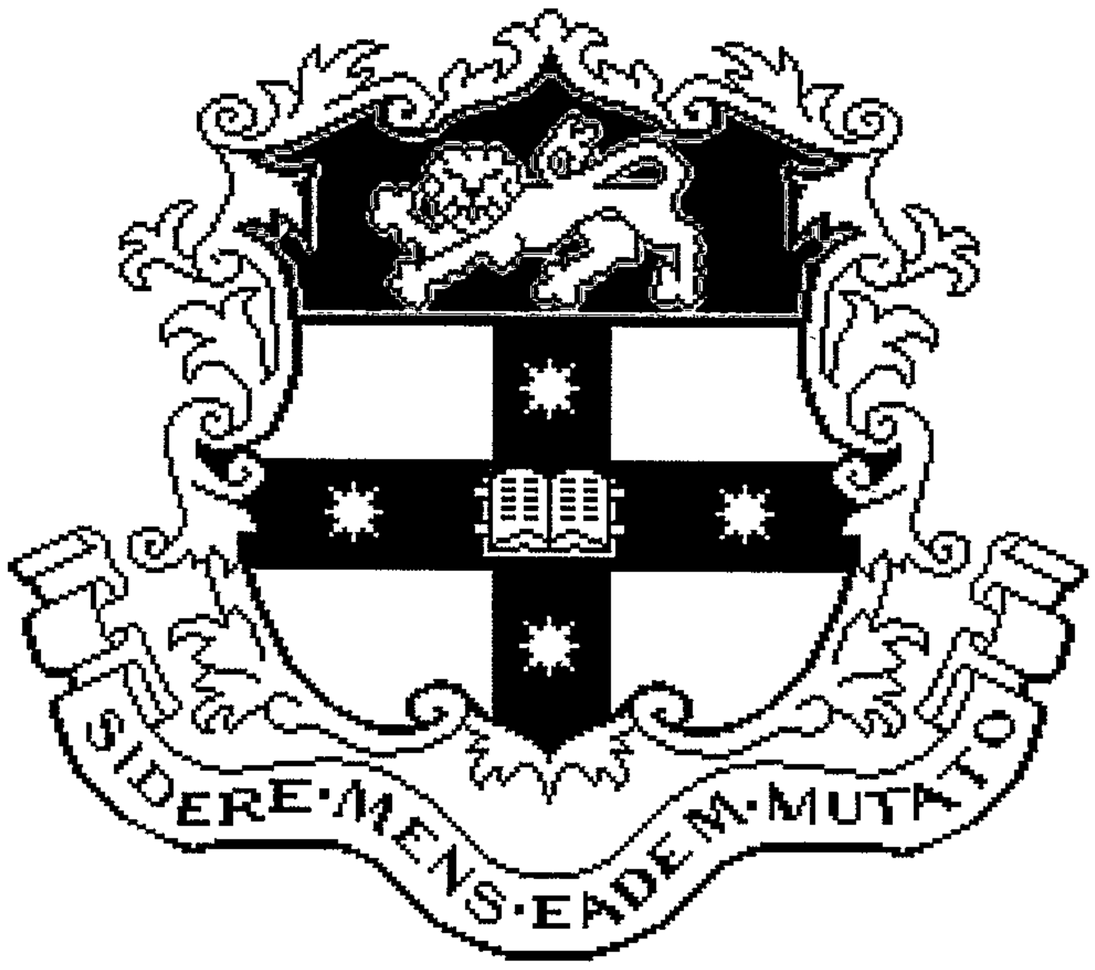
Zerwekh JE, Sakhall K, Pak CYC. Short-term 1,25 dihydroxyvitamin D_3 administration raises serum osteocalcin in patients with postmenopausal osteoporosis. J Clin Endocrinol Metab 1985; 60 : 615-617.

Zerwekh JE, Morris AC, Padalino PK, Gottschalk F, Pak CYC. Fluoride rapidly and transiently raises intracellular calcium in human osteoblasts. J Bone Miner Res 1990; 5 (Suppl 1): 131-136.

Zerwekh JE, Antich PP, Sakhaee K , Prior J, Gonzales J, Gottschalk F, Pak CYC. Lack of deleterious effect of slow-release sodium fluoride treatment on cortical bone histology and quality in osteoporotic patients. Bone Miner 1992; 18: 65-76.

Zipkin I The inorganic composition of bones and teeth. In: Biological Calcification. Schraer, H., Ed. New York, Appleton-Century-Crafts, 1970, p69.

4. Manuscript



The effect of fluoride administration on rat serum osteocalcin expression during orthodontic tooth movement

Abstract

Osteocalcin is a bone-protein that has previously been used as a marker of bone turnover. The precise role of this protein in bone remodelling has as yet not been fully elucidated, however, it has been shown to be instrumental in the activation of osteoclastic bone resorption via its effect on osteoblasts. Fluoride influences bone growth by acting as a mitogenic agent for osteoblasts. This study utilises a rodent model to determine the effects of fluoride administration on systemic osteocalcin during orthodontic tooth movement. Thirty-two, eight week old, female Wistar rats were divided into four groups for this experiment. The first experimental group of rats (n=10) were fed a normal laboratory diet and distilled water (MilliQ). The second experimental group of rats (n=10) had the same diet with the addition of fluoridated water. The third (n=6) and fourth (n=6) groups were used as control groups. These did not undergo orthodontic tooth movement and were given fluoridated (100ppm NaF) and non-fluoridated water *ad libitum*. Both fluoridated and non-fluoridated experimental samples had 100g NiTi coil springs secured to the lower incisor and left first molar tooth to instigate orthodontic tooth movement for a period of 14 days.

Phlebotomy was performed via the lateral tail vein prior to application of the orthodontic appliance and final blood samples were obtained via cardiac puncture following sacrifice two weeks later. Serum was isolated from the samples following centrifuging at each stage of phlebotomy and an enzyme-linked immunosorbent assay (ELISA) was subsequently performed to determine osteocalcin levels in the various sample groups.

Orthodontic tooth movement using high, continuous forces was found to have a positive, statistically significant correlation with serum osteocalcin expression in the rodent model. The use of fluoride as a variable was found to increase the mean osteocalcin concentration, however, this was not statistically significant.

***Keywords*:** Osteocalcin, fluoride, cementum, tooth movement, ELISA.

Introduction

Osteocalcin is a non-collagenous bone-, dentine-, and cementum-specific protein that comprises about 2% of the total protein in bone[1]. Osteocalcin is a calcium-binding peptide synthesized almost exclusively by osteoblasts and odontoblasts[2] .

The distribution of osteocalcin in the mammalian body is limited to the calcified tissues such as bone, tooth cementum, and dentine[3]. It is distinguished by its small size (5800 daltons) and the presence of gamma-carboxy-glutamic acid (Gla). In the presence of ionic calcium, the Gla residues allow a specific conformational change in the protein, which in turn promotes osteocalcin binding to bone mineral and subsequent accumulation in bone matrix. Osteocalcin binds weakly to free calcium ions and more tightly to calcium in hydroxyapatite crystals. Spectroscopic studies suggest that the molecule undergoes a conformational shift when associated with hydroxyapatite, partly as a result of electrostatic attraction between specific anionic residues in the protein and the calcium ions embedded in the face of the crystal[4]. These unique structural features of osteocalcin that dictate the association with calcium ions and Ca^{2+} mineral surfaces may confer on this protein the ability to modulate the mineral dynamics of alveolar bone and cementum.

The proposition that osteocalcin may function as a negative regulator for mineral apposition is supported by the observation that purified osteocalcin inhibits the spontaneous conversion of brushite to hydroxyapatite and inhibits the formation of hydroxyapatite crystals in supersaturated calcium/phosphate solutions *in vitro*[5,6].

Through this high affinity for calcium ions and hydroxyapatite, osteocalcin was thought to be important in the regulation of mineral apposition in calcified tissues[7].

However, warfarin treatment to prevent gamma-carboxylation and hence accumulation of osteocalcin in mineralized matrix failed to demonstrate a defect in mineralization[3,8].

Glowacki *et al.,* (1991) used subcutaneous apatite in the rat model with and without osteocalcin treatment to demonstrate that osteocalcin might function as a matrix signal in the recruitment and differentiation of bone-resorbing cells[9]. The precise role of this protein in bone remodelling has as yet not been fully elucidated; however, it has been shown to be instrumental in the activation of osteoclastic bone resorption via its effect on both osteoblasts and osteoclasts.

Liggett *et al.* (1994) provided support for this hypothesis with the observation that osteocalcin promotes differentiation of osteoclast progenitor cells from murine long-term bone marrow cultures in the presence of colony-stimulating factor (M-CSF) and granulocyte-macrophage stimulation factor (GM-CSF), but not in the absence of these factors. M-CSF has been shown to be a crucial factor in osteoclastogenesis[10].

In vitro experiments have proved that osteocalcin is chemotactic for monocytes and osteoclasts in addition to activation of initial adherence of pre-osteoclasts in a dose-dependent manner[11].

Ducy *et al.* (1996) generated osteocalcin-deficient mice using embryonic stem cell technology and subsequent histomorphometry indicated that the lack of osteocalcin led to an increase in bone formation and an increase in osteoclast number in the bones of the osteocalcin-deficient mice[12].

To the same end, Wolf (1996) used mice that lacked the genes for osteocalcin to study the function of the bone protein[13]. These osteocalcin-deficient mutants developed increased bone density and thickness. Their osteoblasts deposited more bone matrix than those of the wild-type mice. This too, infers that osteocalcin is a negative regulator of bone formation.

Serum osteocalcin reflects the 10-40% of osteocalcin produced that is not incorporated into the bone matrix. The majority of osteocalcin secreted by the osteoblast is deposited in extracellular bone matrix and serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite in the mineralized tissues. Comparable levels of osteocalcin are found in both serum and plasma samples of humans and animals[5] .

The normal degree of coupling between bone formation and resorption is disturbed in orthodontic tooth movement[14] . This occurs at an early stage at sites of pressure and later in sites of tension.

These imbalances facilitate tooth relocation by removing bone ahead of the tooth and depositing it behind. In this respect, this quantitative uncoupling of bone remodelling is similar to the process of osteoporosis.

The anabolic action of fluoride is well established. The osteogenic property of increasing bone mass through stimulating bone formation is the basis for the previous use of fluoride as a therapeutic agent in osteoporosis[15-18].

Fluoride influences bone growth by acting as a mitogenic agent for osteoblasts[19] and may also act as an inhibitory stimulus to osteoclasts, although this is less well understood due to the possible side-effect of fluoride-induced secondary hyperparathyroidism[20]. It has been shown that osteoblast precursors are more sensitive to fluoride than mature osteoblasts and that the *in vivo* effects of fluoride on bone formation may be mediated by stimulating proliferation and differentiation of committed osteoblast precursors in cancellous bone[21].

Fluoride also alters the composition and crystalline state of mineral with the substitution of hydroxyl ions by fluoride ions. Fluoroapatite has a greater crystallinity, larger crystal size, and lower aqueous solubility than hydroxyapatite[22]. Moreover, a mixture of fluoroapatite and hydroxyapatite has been shown to be less soluble than either component individually[23]. Hence, changes in apatite crystallinity could explain the inhibition of mineralised tissue resorption seen after fluoride treatment.

There is *in vitro* evidence that fluoride decreases the number of resorption lacunae as well as the amount of bone resorbed per osteoclast[24]. Increased resistance to dissolution by osteoclastic enzymes as well as high levels of fluoride liberated from fluoride-containing bone during the resorptive process might inhibit osteoclastic activity and explain this effect of fluoride[25].

The aim of this study was to investigate the serum osteocalcin levels that could be elicited with constant orthodontic force levels from a superelastic coil spring applied to rat molar teeth using fluoride as a variable.

Null Hypotheses

1. That there is no discernible difference in the measurements of serum osteocalcin in rats subjected to a high fluoride diet compared to those rats limited to distilled water intake following the application of high orthodontic forces to mandibular molar teeth.
2. That there will be no increase in systemic osteocalcin following the application of high orthodontic forces to mandibular molar teeth in rats.

Materials and Methods

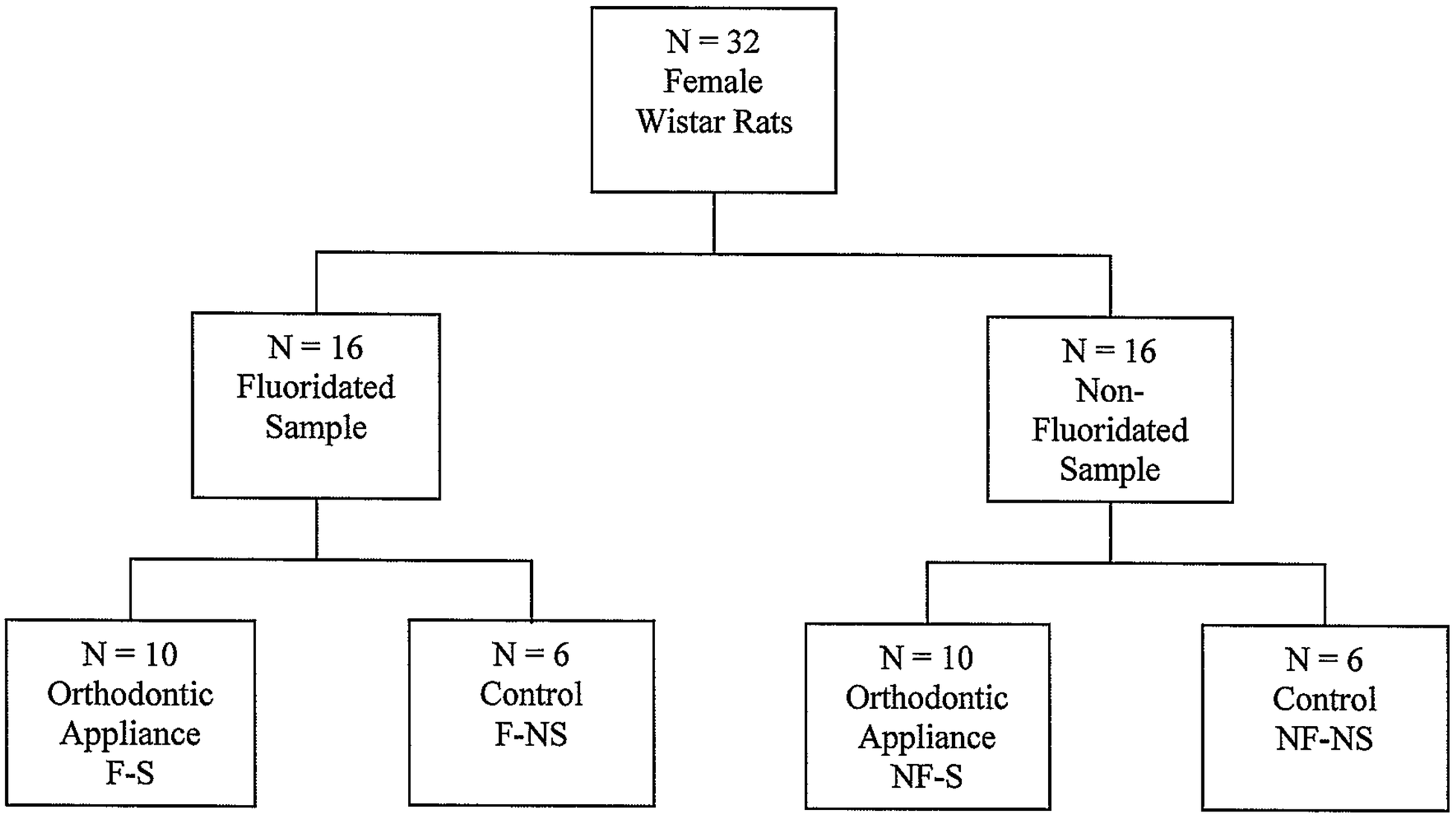
Animals

Thirty-two, 7-8 week old female, Wistar rats weighing approximately 210 g were obtained from the Westmead Animal Holding Facility and conditioned in experimental cages for at least 24 hours before use in experiments (Ethics approval: Westmead Hospital Animal Ethics Committee Project no. 134.12-03) (Figure 1).

The rats were divided into four groups for the purposes of this experiment. The first group of rats (n=10) were fed a standard pellet diet and distilled water (MilliQ) *ad libitum*. The second group of rats (n=10) consumed the same diet with the addition of fluoridated water (100ppm NaF) *ad libitum*. The third (n=6) and fourth (n=6) groups served as controls. These rats did not undergo orthodontic tooth movement and were given fluoridated and non-fluoridated water *ad libitum* respectively.

Figure 1

NF-NS	= non-fluoridated control (no springs)
NF-S	= non-fluoridated rats with springs
F- NS	= fluoridated control (no springs)
F-S	= fluoridated rats with springs



Appliance placement

A superelastic, fixed, closed coil spring appliance was used to facilitate the mesial movement of the mandibular lower left first molar (GAC International Inc., Central Islip, NY). The orthodontic force thus applied was approximately 100g (Figure 2). The duration of the experimental period was 14 days with no reactivation of the appliance during this time period.

The Nickel Titanium spring was ligated (with a stainless steel ligature) to the central incisor teeth and attached to the first lower left molar using a 3/0 black silk suture. The placement of appliances was performed under general anaesthesia, using Xylazine (10mg/kg) and Ketamine (10mg/kg).

Phlebotomy was performed via the lateral tail vein with a 26-gauge needle prior to application of the orthodontic appliance. Following clotting of the blood sample, centrifugation at 5000 rpm for five minutes was undertaken before serum transfer with further centrifugation at 10,000 rpm for five minutes. The supernatant serum was immediately isolated to avoid haemolysis, and then frozen at -70°C.

Following appliance application, the rodents were permitted to recover from anaesthesia before being placed into separate experimental cages. At the end of the experimental period of 14 days the rodents were euthanased with carbon dioxide asphyxiation before final blood collection via intracardiac sampling.

Sample Analysis

Osteocalcin Assay

An enzyme-linked immunoassay (ELISA) kit was used for the detection of osteocalcin (Biomedical Technologies Inc. Stoughton, MA 02072, USA).

Briefly, the capture antibody (mouse monoclonal anti-osteocalcin) was added to a microtitre plate and incubated overnight at 4°C. Following blocking and wash stages, standard solutions of osteocalcin and the test samples were added. Standard solutions were prepared by serial dilution of the re-hydrated standard osteocalcin provided by the kit manufacturer.

The standard curve obtained from these data was used to determine the concentration of osteocalcin detected in the eluted serum samples analysed in the assay.

Following a wash stage, the detection antibody, murine monoclonal antibody to osteocalcin conjugated to alkaline phosphatase, was applied to each well and incubated for 1hr at room temperature. After washing, 100µl of substrate solution, containing a chromogen was added to each well. The microtitre plate was incubated at room temperature for 24hrs. 100µl of stop solution (1N sulphuric acid) was then applied to each well. The plate was transferred to a plate reader and the absorbance of the colour reaction was measured at 450nm. Two separate ELISA kits were needed to assay all unknown samples in duplicate.

Results

Weight gain

The mean weight of the rats in the F-S, NF-S, F-NS and NF-NS groups were 225 ± 13g, 228 ± 19g, 219 ± 23g and 221 ± 13g respectively. All rats in the two groups that had orthodontic appliances placed under anaesthesia experienced some initial weight loss, which was not fully recovered during the experimental period. At the end of two weeks the non-fluoridated sample (NF-S) with appliances lost 16g and the fluoridated sample with appliances (F-S) lost 28g. Both control groups not receiving orthodontic appliances (F-NS, NF-NS) showed an increase in weight; 32g in the non-fluoridated sham and 21g in the fluoridated sham sample respectively.

Water consumption

Water consumption was monitored throughout the trial period and the average individual consumption of the rats receiving distilled water amounted to 16ml per day. The rats receiving fluoridated water (100 ppm NaF) consumed an average of 10ml per day.

Appliance activation

All closed coil springs retained greater than 200 per cent activation at the end of the experimental period, indicating that there was no significant deactivation of the appliances during this time.

Statistical analysis (Tables 1 and 2)

All statistical analysis was done with the assistance of SPSS (SPSS Chicago 60606, Illinois). Analysis of the raw data revealed there were three significantly aberrant values in the post-mortem samples (NF-S, F-S and NF- NS) which were two, three and four times the mean values for their sample groups respectively. Due to the large isolated deviations of these samples from the mean and their distance from the mean regression line, it seemed reasonable to exclude these values from statistical analysis in a second ANOVA.

The influence of inter-kit variation

Two separate ELISA kits were needed for this experiment and, therefore, the possibility of inter-kit variation interfering with the interpretation of subsequent results was a possibility. Statistical analysis using ANOVA, however, refuted this influence as an obfuscating factor with a p-value of 0.864 (significance was taken as $p<0.05$).

The effect of springs on osteocalcin expression

The orthodontic movement of the rat molar elicited by the appliance resulted in a marked increase in mean osteocalcin levels, which only achieved a level of moderate statistical significance ($p<0.051$) with the univariate analysis of variance. However, if the three aforementioned aberrant values are removed from the analysis, then this variable attains a moderate level of significance ($p<0.03$).

The effect of fluoride on osteocalcin

Osteocalcin expression was found to remain close to baseline levels when control rats from the non-fluoridated group were compared with the fluoridated shams. ANOVA was used for statistical analysis of variables and there was found to be no statistically significant increase in osteocalcin in rats without orthodontic appliances, regardless of fluoride consumption ($p < 0.524$) (Figures 3 and 4).

The effect of fluoride and springs on osteocalcin expression

Although there was a mean increase in osteocalcin concentration associated with the F-S sample, fluoride together with the use of the orthodontic appliance was not found to have a statistically significant interaction with one another on osteocalcin expression overall (Figures 5 and 6).

Discussion

It has previously been shown that fluoride consumption in rats at levels in excess of 50mg/mL has an adverse effect on weight of the animals compared with controls[26], and although this was found to be the case in this study, it is not possible to determine a direct correlation. This weight loss may, however, also be explained by the lower water consumption of the fluoridated samples, due in part perhaps to the poor taste of the medium of fluoride administration.

Osteocalcin is markedly unstable *in vitro*[27, 28] , rapidly degrading in samples at room temperature and at 4° C and is also sensitive to freeze-thaw cycles and haemolysis. Repeated freeze-thaw cycles have been shown to reduce immunoreactivity by up to 40%[29]. Haemolysis decreases apparent osteocalcin concentrations, due to proteolysis by enzymes released from lysis of red blood cells[30]. Given the inherent problems of sample stability and biological variability (including diurnal variation with circadian rhythm), there was an absolute need for continuity in the methodology for sample collection. This included the avoidance of haemolysis and freeze-thaw cycles as well as time-dependent phlebotomy during collection of the samples[31].

Young rats were chosen because it has been shown that there is complete formation of dentine, cementum, alveolar bone and the periodontal ligament at eight weeks in this particular animal model[32]. In addition, young rats have been shown to recover rapidly from the surgical insult in orthodontic appliance placement[33].

Bridges *et al.* (1988) evaluated the effects of age on tooth movement and concomitant mineral density in the treated tissues of the rat[34]. The absolute amount of tooth movement occurring in a cycle and the rate of tooth movement in the late part of the cycle were significantly greater in the younger animals (21-28 days old), while the total time required for a cycle was shorter in the younger animals. The "instantaneous" tooth movement was greater in the young animals than older animals (100 days old) and the control tissue mineral density was lower, suggesting that bone density may play a key role in this phase of the cycle. Mineral densities returned to control levels at

approximately the same rate in the tissues of both groups during the late part of the cycle, but the rate of tooth movement in the adult was less than in the younger animal.

This suggests that the proportions of bone formation to resorption are equivalent at comparable times in both groups, but that absolute amounts of each may be higher in the younger animals.

King and Fischlschweiger (1982) showed that when appliances were loaded with increasing amounts of force, the specific activity of bone resorption stimulators increased progressively[35].

This was highlighted by the study of Ashizawa and Sahar (1998) who moved rat molars mesially with a fixed coil-spring appliance using three different magnitudes of initial tensile force (27, 60 and 136g)[36]. They found little or no relationship between the magnitude of the initial tensile force and the amount of new bone formation in the alveolar wall on the tension side during the initial stage of tooth movement. However, a heavy initial force (136g) produced a wide necrotic area of periodontal ligament, and delayed the bone remodelling that occurred on the pressure side. It was, therefore, decided to use a heavy force of 100g in this present study so that there would be a consistent and constant force during the experimental period, which would maximize the potential for indirectly observing hard tissue turnover. This in turn would increase the possibility of assessing the relationship between this hard tissue turnover and subsequent osteocalcin expression.

Studies that have utilized an antibody against the propeptide of osteocalcin have indicated that osteocytes are the primary source of osteocalcin in human bone, and that it may function by signalling osteoclasts and/or their precursors to initiate bone resorption[8]. Kagayama *et al.* (1997) investigated the expression of osteocalcin in Wistar rats[37]. They found that all cells lining cellular cementum were positive for osteocalcin. They suggested that the osteocalcin expression of cementoblasts forming acellular cementum is similar to that of cells forming cellular cementum as well as osteoblasts and odontoblasts. Consistent with these observations, the cells engaged in the formation of cellular cementum (cementoblasts and cementocytes) were also found to be associated with large amounts of osteocalcin[38]. Bronckers *et al.,* (1994) concluded that cementoblasts and cementocytes of cellular cementum produce osteocalcin and express an osteoblast-like, not an odontoblast-like phenotype[39]. In addition, cells and matrices of surrounding alveolar bone were found to strongly express the bone protein.

Lu *et al.* (1999) applied orthodontic forces (50g) to mandibular rat molars, demonstrating higher distribution of osteoclastic cells on the alveolar bone than on the root surface, indicating that alveolar bone is more readily removed than the root surface[40]. They also showed that cellular cementum may be resorbed more easily than acellular cementum due to its enhanced organic components and low mineralized structure. Hence, the dissolution of tooth structure as occurs during the process of physiologic tooth movement may result in the release of osteocalcin.

Local osteocalcin administration in the orthodontic rat model was found to induce mature osteoclasts on the pressured side of orthodontic tooth movement but not on the tension side[41].

This suggests that osteocalcin has an additive effect on the rate of orthodontic tooth movement through the enhancement of orthodontic tooth movement on the pressured side. In a similar experiment involving the local injection of osteocalcin adjacent to rat molars over a longer period of time, Hashimoto *et al.* (2001) came to the same conclusion[42].

They suggested that osteocalcin might act as a chemoattractant for osteoclast precursor cells and an enhancer of maturation of multinuclear osteoclasts on the pressure side, where it may act in concert with bone resorbing factors (such as macrophage colony-stimulating factor [M-CSF]). This then gives rise to an appropriate microenvironment for osteoclastogenesis. It was shown that there was acceleration of tooth movement following local application of osteocalcin in the early experimental period. Histological examination revealed that this acceleration was due to enhanced recruitment of osteoclasts. In contrast to the early days of the experiment, osteocalcin did not show an additive effect on the rate of orthodontic tooth movement towards the end of the ten-day experimental period.

Previously, it has been shown that the chemotactic activity of osteocalcin for human peripheral monocytes and osteoclast precursor cells displayed a bell-shaped dose-response curve. In addition, the serum osteocalcin level has been observed to increase in the later phase of tooth movement in rats[43]. It is therefore possible that the increase in

serum osteocalcin associated with tooth movement may diminish the effect of exogenous osteocalcin in the later period of tooth movement[42].

King and Keeling (1995) examined alveolar bone turnover and orthodontic tooth movement following appliance decay using closed coil springs in the rat model[43], unlike the present study, which used a continuous force. They observed that 93% of appliance activity was lost at day 16, at which time a second wave of bone remodelling began, with abrupt inhibition of ongoing bone formation and the initiation of a second wave of bone resorption. This was accompanied by a parallel rise in the amount of serum osteocalcin detected. The spike in osteocalcin values reached a nadir at day 22 when their experimentation period was concluded.

They interpreted these data as being predictive of a second wave of bone formation (which did not eventuate due to premature conclusion of the experiment) rather than being possibly being representative of the occurring resorption.

The orthodontic movement of murine teeth by Pavlin and Gluhak-Heinrich (2001) was used to elucidate the response of osteocalcin to mechanical stimuli over a period of 14 days[44]. After 24 hours of applied force, there was an inhibition of osteocalcin expression by 80%, followed by a marked four-fold induction at day two. Osteocalcin expression peaked six-fold at day four, then fell. However, the levels remained over four-fold higher compared with controls at day six. These results suggested relatively early expression of osteocalcin in differentiating osteoblasts and much earlier than the mineralization stage.

In the light of the results from osteocalcin-deficient mice and a proposed suppressive role of osteocalcin in bone formation[12], its up-regulation in the areas of osteoblastic activity in the mouse orthodontic model is consistent with its possible role as a determinant of the rate of mechanically induced bone remodelling.

A role for osteoblastic involvement in the control of bone resorption was hypothesized by Rodan and Martin (1981)[45]. The hypothesis suggested that resorbing agents, such as PTH and prostaglandins, induce a shape change in osteoblasts (via cAMP and Ca^{2+}), which uncovers matrix for osteoclastic digestion, further enhancing subsequent resorption by releasing osteocalcin. This interdependent relationship of osteoblasts and osteoclasts in the orchestration of hard tissue remodeling may, therefore, be partly facilitated by osteocalcin.

There are also significant differences in the genetic responses of cementoblasts and osteoblasts to mechanical stimulation. Pavlin *et al.* (2001) found that there was only a moderate stimulatory treatment effect on the expression of osteocalcin expression in cementoblasts (similar induction of osteocalcin expression in both cementoblasts of acellular cementum and cementoblasts of cellular cementum), compared to a six-fold induction in osteoblasts[46].

Hence it was postulated that orthodontic tooth movement using heavy forces lead to the increased expression of serum osteocalcin from local sources including cementum and alveolar bone.

In this study, osteocalcin expression was indeed increased by the use of heavy orthodontic forces. The precise tissue of origin of the osteocalcin was, however, unable to be determined by the present methodology. The osteocalcin may have originated from local cementum degradation, alveolar bone dissolution or from local production by osteoblasts associated with the periodontal ligament and cementoblasts.

Singer *et al*. (1967) found that indirect resorption occurred in the absence of periodontal ligament hyalinization during tooth movement induced in fluoride-treated rats[47]. There was found to be considerably less bone loss and osteoclastic activity in the fluoridated rats than in the non-fluoridated group, thus interfering with experimental tooth movement in the former. Much smaller forces in the form of elastomeric separating modules were used in this particular study than were used in the present study and this may explain the absence of hyalinization observed.

Cheng and Bader (1990) concluded that fluoride affects rat cortical and cancellous bone differently and that fluoride increases rat cancellous bone volume through trabecular thickening in a dose-dependent manner[48]. This is most probably due to a combination of: reduced bone resorption, increased bone mineral packing density and increased size of individual bone mineral crystallite. This mirrors the findings in human subjects[16].

The lack of intracortical (Haversian) remodelling in the rat may explain any difference in bone mass when compared with humans[49].

Fluoride incorporation into the skeleton is largely dependent upon remodelling (formative) surfaces, and very little response should be expected in cortical bone in rats. It has also been demonstrated that fluoride is generally more readily incorporated into cancellous bone than cortical bone[50] which may be seen to directly impact upon orthodontic tooth movement.

The evolution of bone changes induced by fluoride was investigated in lambs by Chavassieux *et al.* (1991)[51]. Together with an increase in bone formation it was found that serum osteocalcin was twice as high in treated animals as compared to controls.

The effect of mitogenic doses of sodium fluoride on osteocalcin in the rat model was investigated by Ohta *et al.,* (1995), who found that serum osteocalcin levels were significantly increased together with mineral apposition rate and bone formation rate in fluoride treated rats when compared to controls[52]. This was also seen in the present study, where mean increases in osteocalcin concentration were observed in the fluoridated, orthodontic appliance group. These increases were not, however, statistically significant and may have been a function of the small sample size.

If fluoride could indeed decrease the resorption of mineralized tissues then it might be expected that local expression of osteocalcin would reduce. If local osteocalcin

expression was expressed in a systemic concentration increase then this is not reflected in these aforementioned results.

Ingram *et al.* (1994) showed that the presence of an osteocalcin-rich matrix increased the recruitment and binding of osteoclasts and, therefore, osteocalcin was postulated to be a site-specific regulator of bone remodelling[53]. As fluoride has been shown to be mitogenic to osteoblasts, then it is possible that the observed fluoride-induced increase in serum osteocalcin during this study may just be a biological representation of this anabolic process. Although there was no statistical significance with respect to osteocalcin expression in orthodontically-treated rodents exposed to fluoride, this may have been a function of the reduced power of the sample size and not necessarily, because of an absent interaction.

A further factor which may have affected the results is the lack of pre-treatment with fluoride. Concomitant exposure to fluoride during the period of experimentation may have reduced the potential for fluoride incorporation into the mineralized tissues and, therefore, reduced the manifestation of its protective mineralized tissue properties.

On the basis of these results Null Hypothesis No. 1 (that there is no increase in OC expression at a systemic level upon application of an orthodontic force in a rodent model) must be rejected. Null Hypothesis No. 2, (that the systemic intake of fluoride does not increase serum OC expression in a rodent model following orthodontic force application), however, cannot be rejected on the basis of the results obtained from this study.

Conclusions

The systemic expression of osteocalcin was found to be positively correlated with the local event of orthodontic tooth movement in this rodent model. Although the addition of fluoride as a variable was found to result in a mean increase in systemic osteocalcin expression, this increase was not found to be statistically significant.

The source of the increased osteocalcin observed as a result of the applied orthodontic forces was not able to be ascertained with the current methodology and therefore it was not possible to further clarify the nebulous role that this bone protein plays during the complex orchestration of mineralized tissue turnover. Further understanding of the biological implications of increased osteocalcin expression may therefore require further research into the *local* expression of this protein in the gingival crevice during orthodontic movement. Fluoride has previously been shown to have a protective role with respect to hard tissue demineralization and although this was not evident in the present study, pre-treatment of the experimental samples with fluoride may have provided more insight into the nature of this property with respect to osteocalcin expression.

This research project was generously supported by grants from The Australian Society of Orthodontists' Research Fund and The University of Sydney Faculty of Dentistry Research Grant Scheme.

4.1 Manuscript References

1. Knepper-Nikolai B, Reinstorf, Hofinger K, Flade K, Wenz R, Pompe W. Influence of osteocalcin and collagen I on the mechanical and biological properties of Biocement D. Biomol Engineering 2002; 19: 227- 231.

2. Fassbender WJ, Steinhauer B, Stracke H, Schumm-Draeger PM, Usadel KH. Validation of a new automated immunoassay for measurement of intact osteocalcin. Clin Lab. 2002; 48: 31-38.

3. Bronckers ALJJ, Price PA, Schrijivers A, Bervoets TJM, Karsenty G. Studies of osteocalcin function in dentine formation in rodent teeth. Eur J Oral Sci 1998; 106: 795-807.

4. Cole DE, Hanley DA. Osteocalcin. In: Hall, BK (Ed.), Bone Matrix and Bone Specific Products. CRC Press, London, UK, pp 239-294.

5. Hauschka PV, Lian JB, Cole C, Gundberg CM, Osteocalcin and MGP: vitamin K-dependent proteins in bone. Physiol Rv 1989; 69: 990-1047.

6. Price PA. Gla-containing proteins of bone. Connect Tissue Res 1989; 21: 51-60.

7. Price PA, Williamson MK, Lothringer JW. Origin of the vitamin-K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem. 1981; 256: 12760-12766.

8. Robey PG. Vertebrate mineralized matrix proteins: structure and function. Conn Tiss Res 1996; 35: 185-190.

9. Glowacki J, Rey C, Glimcher MJ, Cox KA, Lian J. A Role for Osteocalcin in Osteoclast Differentiation. J Cell Biochem 1991; 45: 292-302.

10. Liggett WH, Lian JB, Greenberger JS, Glowacki J. Osteocalcin promotes differentiation of osteoclast progenitors from murine long-term bone marrow cultures. J Cell Biochem 1994; 55: 190-199.

11. Chenu C, Colucci S, Grano M, Zigrino P, Baratollo R, Zambonin G, Baldini N, Vergnaud P, Delmas PD, Zallone AZ. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signalling in human osteoblast-like cells. J Cell Biol 1994; 4: 1149-1158.

12. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C. Increased bone formation in osteocalcin deficient mice. Nature 1996; 382: 448-452.

13. Wolf G. Function of the bone protein osteocalcin: definitive evidence. Nutr Rev 1996; 54: 332-333.

14. Melsen B. biological reaction of alveolar bone to orthodontic tooth movement. Angle Orthod 1999; 69: 151-158.

15. Lau KH, Baylink DJ. Molecular mechanism of action of fluoride on bone cells. J Bone Miner Res. 1998; 13: 1660-1667.

16. Compston, J. Mechanisms of Bone Loss and Gain in Untreated and Treated Osteoporosis. Endocrine 2002; 17: 21-27.

17. Pak CYC, Zerwekh JE, Antich P. Anabolic effects of fluoride on bone. Trends Endocrinol Metab 1995; 6: 229-234.

18. Riggs BL, Hodgson SF, Eastell R. The Role of Sodium Fluoride in the Treatment of Osteoporosis. In: Clinical Disorders of bone and Mineral Metabolism. Eds Kleerekoper M and Krane SM. Mary Ann Liebert Inc Publishers NY 1989.

19. Farley JR, Werdegal JE, Baylink DJ. Fluoride directly stimulates proliferation and alkaline phosphatase activity in bone-forming cells. Science. 1983; 222: 330-332.

20. Bonjour JP, Caverzasio J, Rizzoli R. Effect of fluoride on bone cells. Res Clin Forums 1993; 15: 9-12.

21. Kassem M, Mosekilde L, Eriksen EF. Effects of fluoride on human bone cells in vitro: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. Eur J Endocrinol 1994; 130: 381-386.

22. Posner AS, Eanes ED, Harper RA, Zipkin I. X-Ray diffraction analysis of the effect of fluoride on human bone apatite. Arch Oral Biol. 1963; 8: 549-570.

23. Grynpas MD, Cheng P-T. Fluoride reduced the rate of dissolution of bone. Bone Miner 1988; 5: 1-9.

24. Okuda A, Kanehisa J, Heersche JNM. The effects of sodium fluoride on the resorptive activity of isolated osteoclasts. J Bone Miner Res 1990 (suppl 1): S115-S120.

25. Melsen F, Eriksen EF, Mosekilde L. Clinical Aspects of Fluoride in Bone. In: Fluoride in Dentistry 2nd edition. Fejers O, Ekstrand J, Burt BA, Eds. Munksgaard 1996.

26. Turner CH, Hinckley WR, Wilson ME, Zhang W, Dunipace AJ. Combined effects of diets with reduced calcium and phosphate and increased fluoride intake on vertebral bone strength and histology in rats. Calcif Tiss Int 2001; 69: 51-57.

27. Garnero P, Grimaux M, Seguin P, Delmas PD. Characterization of immunoreactive forms of human osteocalcin generated in vivo and in vitro. J Bone Miner Res 1994; 9: 255-64.

28. Swaminatham R. Biochemical markers of bone turnover. Clinic Chim Acta 2001; 313: 95-105.

29. Gundberg CM, Wilson PS, Gallop PM, Parfitt AM. Determination of osteocalcin in human serum: results with two kits compared with those by a well-characterized assay. Clin Chem 1985; 31: 1720-1723.

30. Tracy RP, Andrianorivo A, Riggs BL, Mann KG. Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. J Bone Miner Res 1990; 5: 451-461.

31. Markowitz ME, Gundberg CM, Rosen JF. The circadian rhythm of serum osteocalcin concentrations: effects of 1,25 dihydroxyvitamin D administration. Calcif Tissue Int 1987; 40: 179-183.

32. Matias MA, Li H, Young WG, Bartold PM. Immumohistochemical localization of fibromodulin in the periodontium during cementogenesis and root formation in the rat molar. J Perio Res 2003; 38: 502-507.

33. King GJ, Latta L, Rutenberg J, Ossi A, Keeling SD. Alveolar bone turnover and tooth movement in male rats after removal of orthodontic appliances. Am J Orhod Dentofac Orthop 1997; 111: 266-275.

34. Bridges T, King G, Mohammed A. Effect of age on tooth movement and mineral density in alveolar tissues of the rat. Am J Orthod Dentofac Orthop1988;93: 245 – 250.

35. King GJ, Fischlschweiger W. the effect of force magnitude on extractable bone resorptive activity and cemental cratering in orthodontic tooth movement. J Dent Res 1982; 61: 775-779.

36. Ashizawa Y, Sahara N. Quantitative evaluation of newly formed bone in the alveolar wall surrounding the root during the initial stage of experimental tooth movement in the rat. Arch Oral Biol 1998; 43: 473-484.

37. Kagayama M, Li HC, Zhu J, Sasano Y, Hatakeyama Y, Mizoguchi I. Expression of osteocalcin in cementoblasts forming acellular cementum. J Perio Res 1997; 32: 273-278.

38. De Vries IG, Coomans D, Wisse E. Immunocytochemical localization of osteocalcin in human and bovine teeth. Calcif Tiss Int 1988; 43: 128-130.

39. Bronckers ALJJ, Farach-Carson MC, Van Waveren E, Butler WT. Immunolocalization of Osteopontin, Osteocalcin, and Dentine Sialoprotein during dental root formation and early cementogenesis in the rat. J Bone Miner Res. 1994; 9: 833-841.

40. Lu L-H, Lee K, Imoto S, Kyomen S, Tanne K. Histological and histochemical quantification of root resorption incident to the application of intrusive force to rat molars. Eur J Orthod 1999; 21: 57-63.

41. Kobayashi Y, Takagi H, Sakai H, Hashimoto F, Mataki S, Kobayashi K, Kato Y. Effects of local administration of osteocalcin on experimental tooth movement. Angle Orthod 1998; 68: 259-266.

42. Hashimoto F, Kobayashi Y, Matakai S, Kobayashi K, Kato Y, Sakai H. Administration of osteocalcin accelerates orthodontic tooth movement induced by a closed coil spring in rats. Eur J Orthod 2001; 23: 535-545.

43. King GJ, Keeling SD. Orthodontic bone remodelling in relation to appliance decay. Angle Orthod 1995; 2: 129-140.

44. Pavlin D, Gluhak-Heinrich J. Effect of loading on periodontal cells. Crit Rev Oral Biol Med 2001; 12: 414-424.

45. Rodan GA, Martin TJ. Role of osteoblasts in hormonal control of bone resorption- a hypothesis. Calcif Tissue Int. 1981; 33: 349-351.

46. Pavlin D, Zadro R, Gluhak-Heinrich J. Sequence of stimulation of osteoblast-associated genes in vivo during mechanically-induced osteogenesis: parallel stimulation of osteocalcin and type- I collagen. Connect Tissue Res 2001; 42: 345-351.

47. Singer J, Furstman L, Bernick S. A histologic study of the effect of fluoride on tooth movement in the rat. Am J Orthod 1967; 53: 296-308.

48. Cheng PT, and Bader SM. Effects of fluoride on rat cancellous bone. Bone Miner. 1990; 11: 156-161.

49. Giavaresi G, Fini M, Gnudi S, Mongiorgi R, Ripamonti C, Zati A, Giardino R. The mechanical properties of fluoride-treated bone in the ovariectomized rat. Calcif Tissue Int, 1999; 65: 237-241.

50. Zerwekh JE, Antich PP, Sakhaee K , Prior J, Gonzales J, Gottschalk F, Pak CYC. Lack of deleterious effect of slow-release sodium fluoride treatment on cortical bone histology and quality in osteoporotic patients. Bone Miner 1992; 18: 65-76.

51. Chavassieux P, Pastoureau P, Boivin G, Chapuy MC, Delmas PD, Meunier PJ. Fluoride –induced bone changes in lambs during and after exposure to sodium fluoride. Osteopor Int. 1991; 2: 26-33.

52. Ohta T, Werdegal JE, Matsuyama T, Baylink DJ, Lau KH. Phenytoin and fluoride act in concert to stimulate bone formation and to increase bone volume in rats. Calcif Tiss Int 1995; 56: 390-397.

53. Ingram, RT, Park YK, Clarke BL, Fitzpatrick LA. Age- and Gender-related Changes in the Distribution of Osteocalcin in the Extracellular Matrix of Normal Male and Female Bone. Possible involvement of Osteocalcin in Bone Remodelling. J Clin Invest 1994; 93: 989-997.

FIGURES

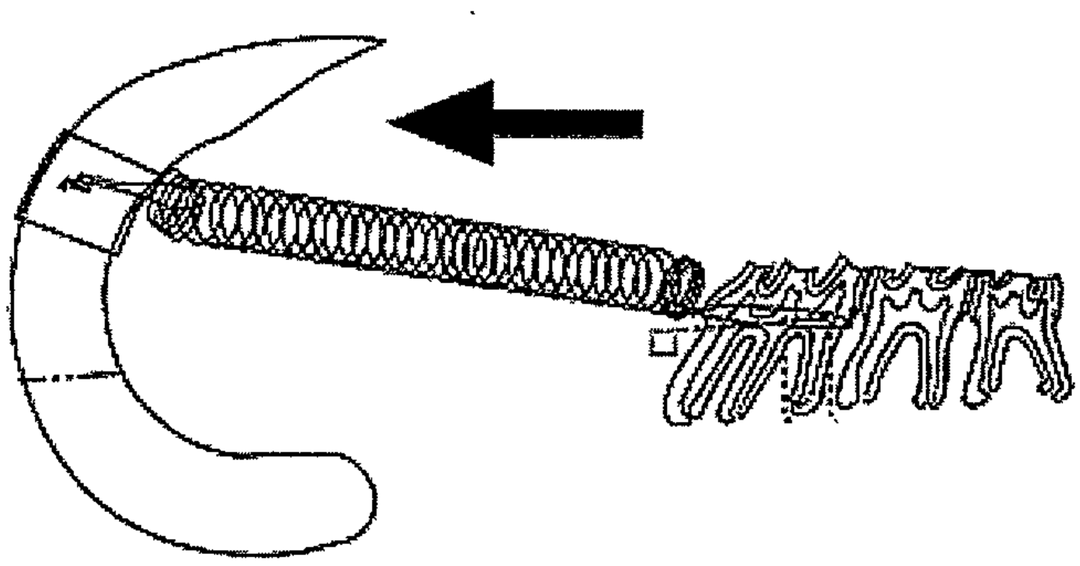


Figure 2. Diagrammatic representation of the orthodontic appliance applied to the mandibular incisors and rodent first molar teeth.

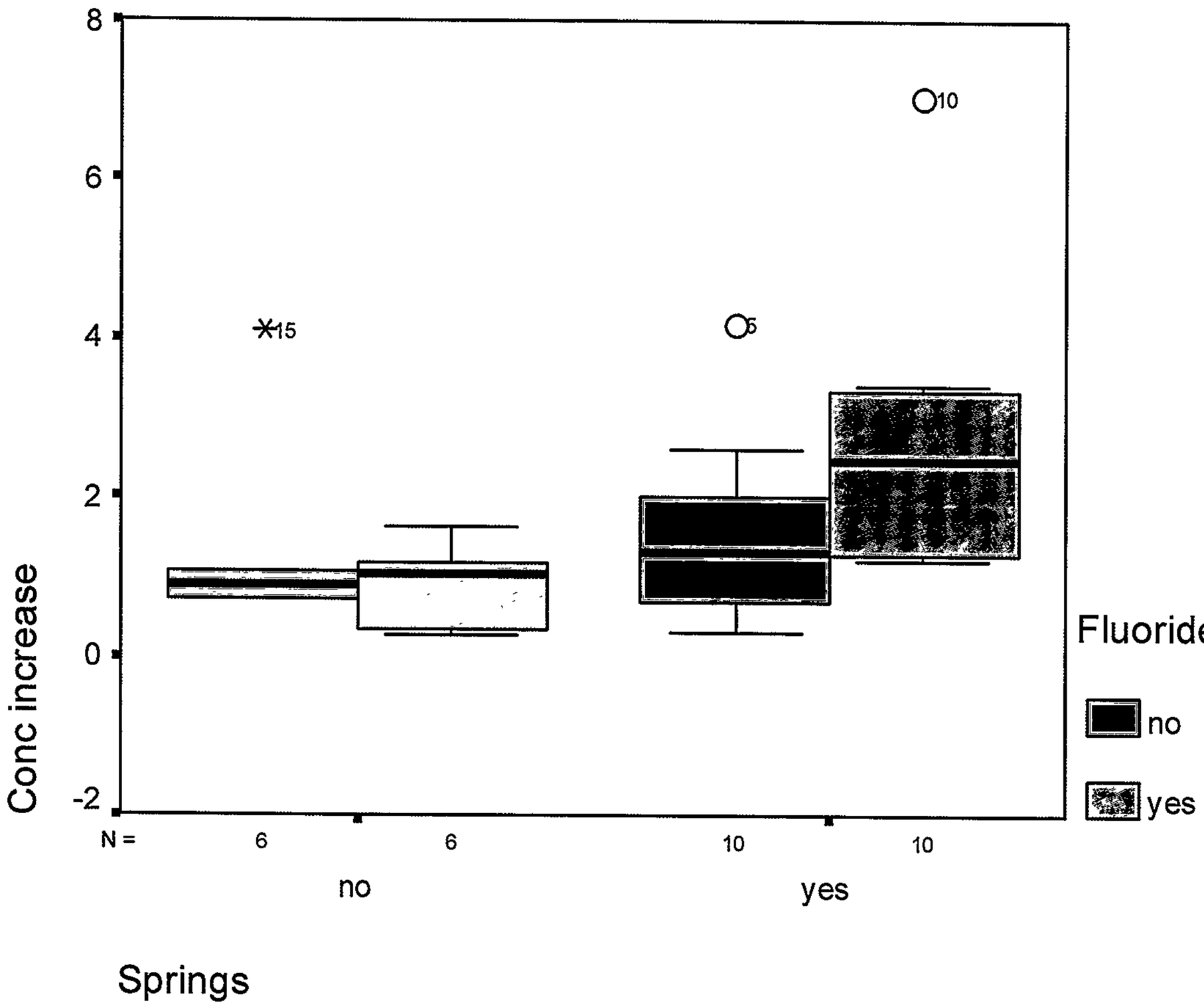


Figure 3. THE EFFECT OF FLUORIDE ON OSTEOCALCIN CONCENTRATION

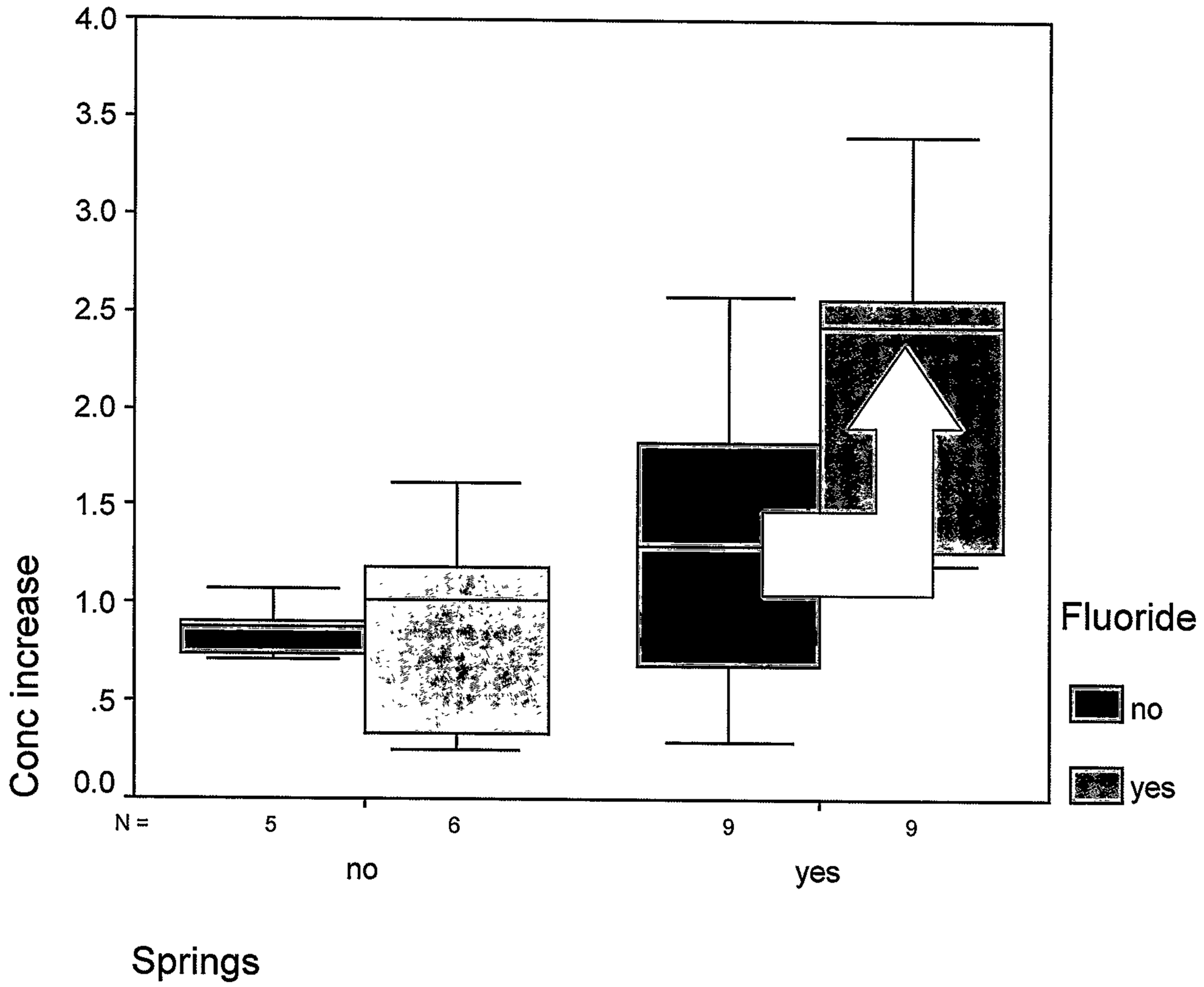


Figure 4. THE EFFECT OF FLUORIDE ON OSTEOCALCIN CONCENTRATION (FOLLOWING REMOVAL OF THREE ABERRANT VALUES).

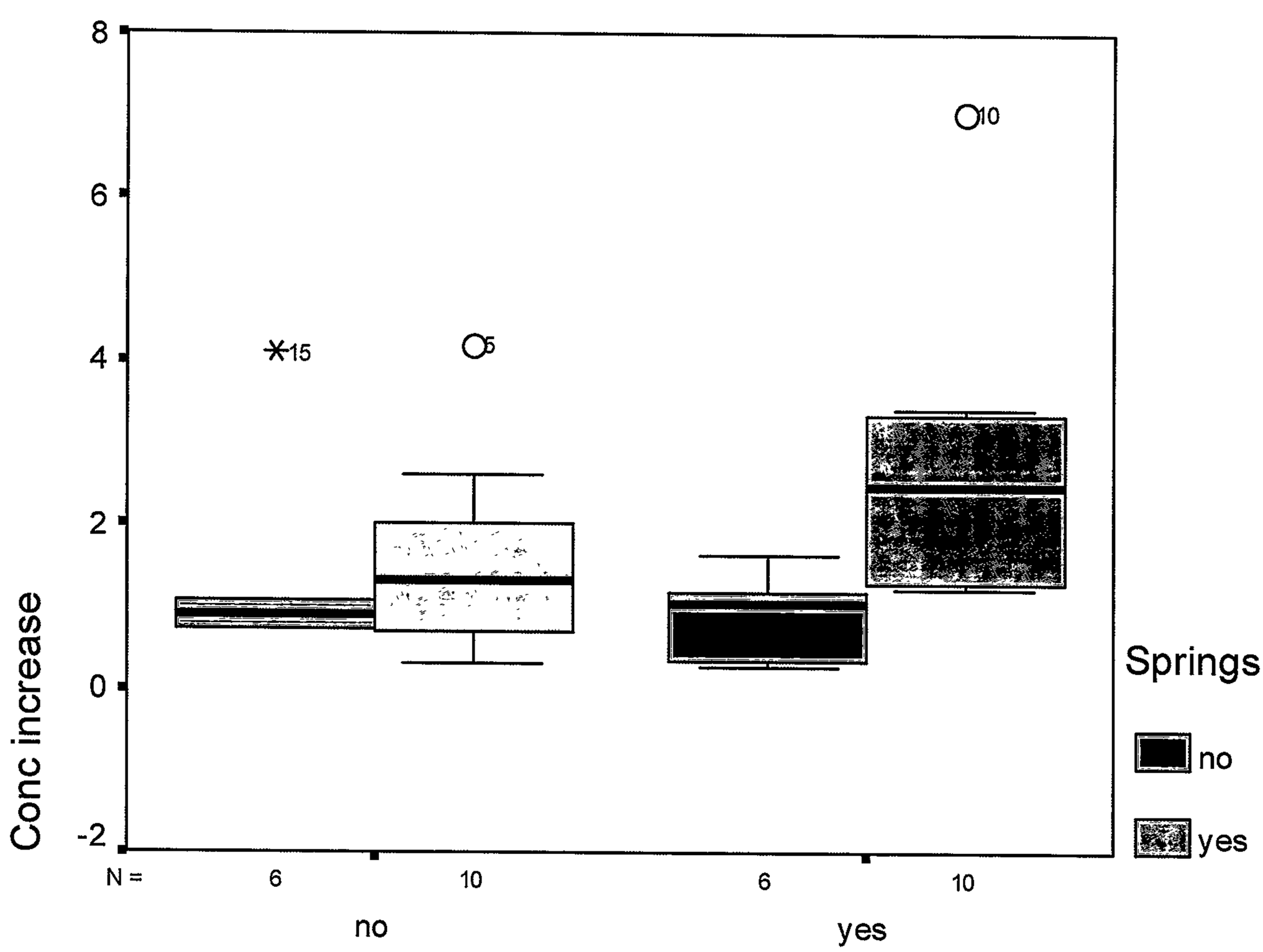


Figure 5. THE EFFECT OF SPRINGS ON OSTEOCALCIN CONCENTRATION

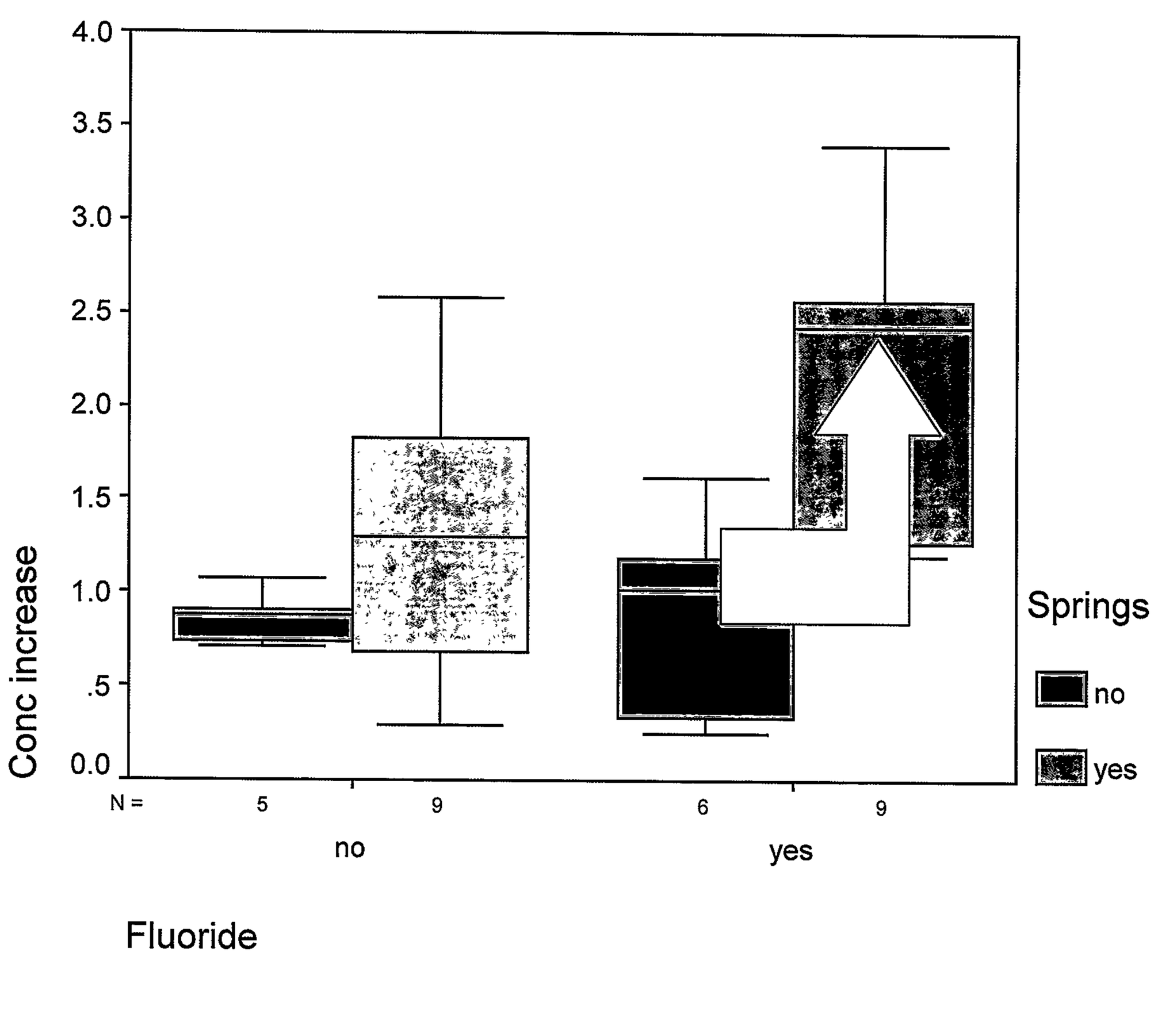


Figure 6. THE EFFECT OF SPRINGS ON OSTEOCALCIN CONCENTRATION (FOLLOWING REMOVAL OF THREE ABERRANT VALUES)

Table 1. Statistical Analysis of all Data

Tests of Between-Subjects Effects

Dependent Variable: DCC Conc increase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.289[a]	3	4.763	2.728	.063
Intercept	81.089	1	81.089	46.444	.000
FL	.727	1	.727	.416	.524
SP	7.276	1	7.276	4.167	.051
FL * SP	4.903	1	4.903	2.808	.105
Error	48.887	28	1.746		
Total	163.110	32			
Corrected Total	63.175	31			

a. R Squared = .226 (Adjusted R Squared = .143)

Table 2. Statistical Analysis with three aberrant values removed

Tests of Between-Subjects Effects

Dependent Variable: DCC Conc increase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9.013[a]	3	3.004	6.347	.002
Intercept	47.119	1	47.119	99.534	.000
FL	1.595	1	1.595	3.369	.078
SP	5.132	1	5.132	10.840	.003
FL * SP	1.334	1	1.334	2.818	.106
Error	11.835	25	.473		
Total	79.531	29			
Corrected Total	20.848	28			

a. R Squared = .432 (Adjusted R Squared = .364)

Legend:
Fl- Fluoridated sample DCC- Osteocalcin
SP- Presence of springs

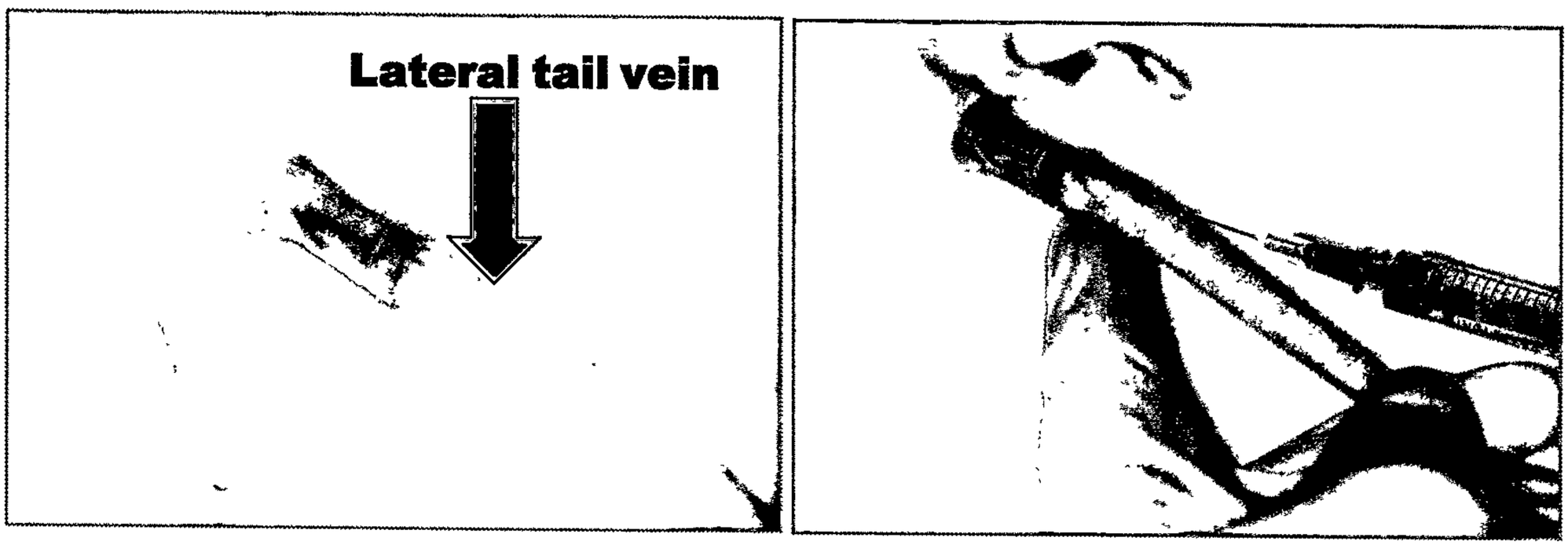


Figure 7- Repeat blood samples were taken at the same time of day due to diurnal variation in osteocalcin concentration (Lee *et al.*, 2000)

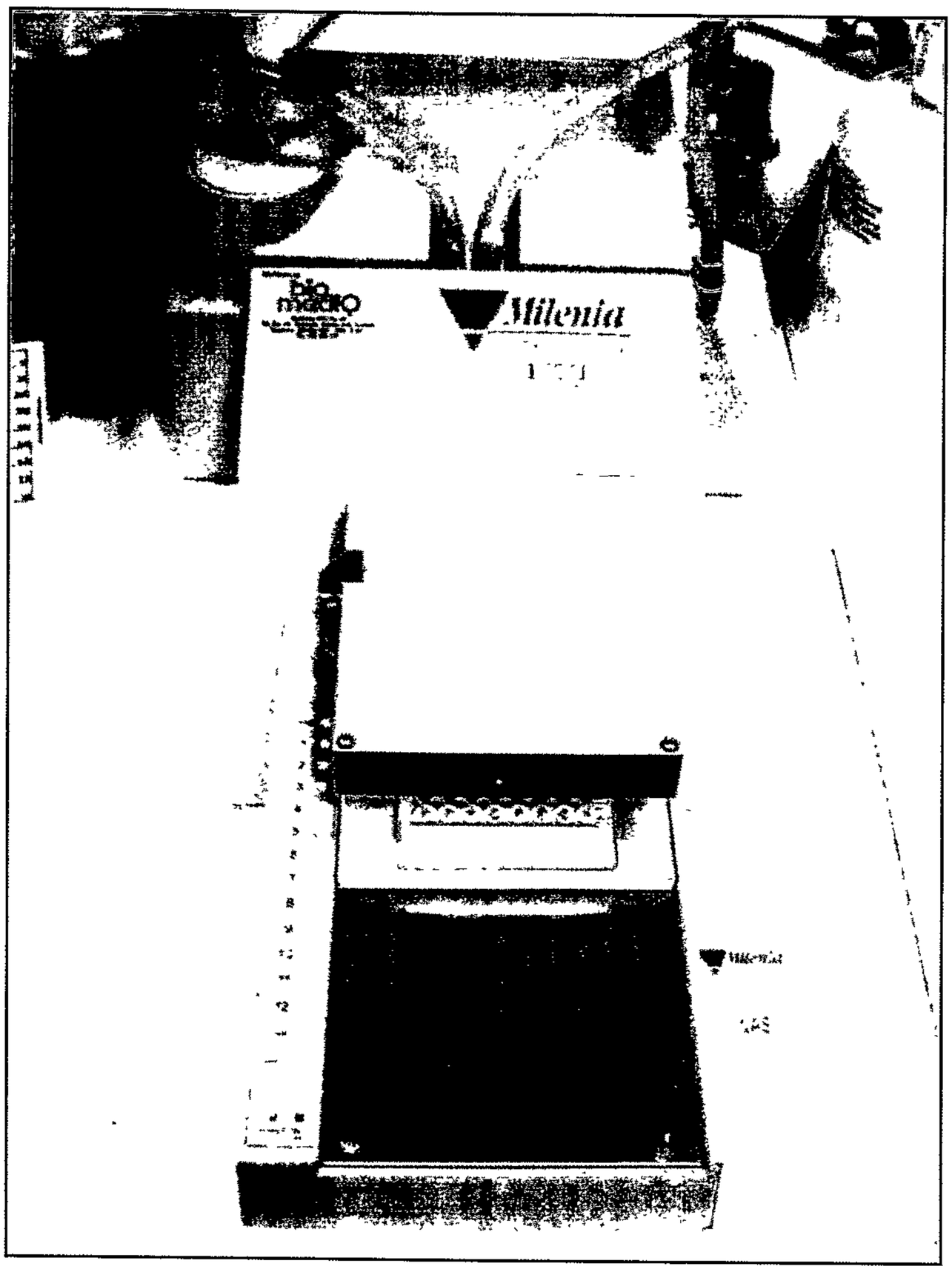


Figure 8- Plate reader preparing to read absorbance of ELISA wells at 450nm

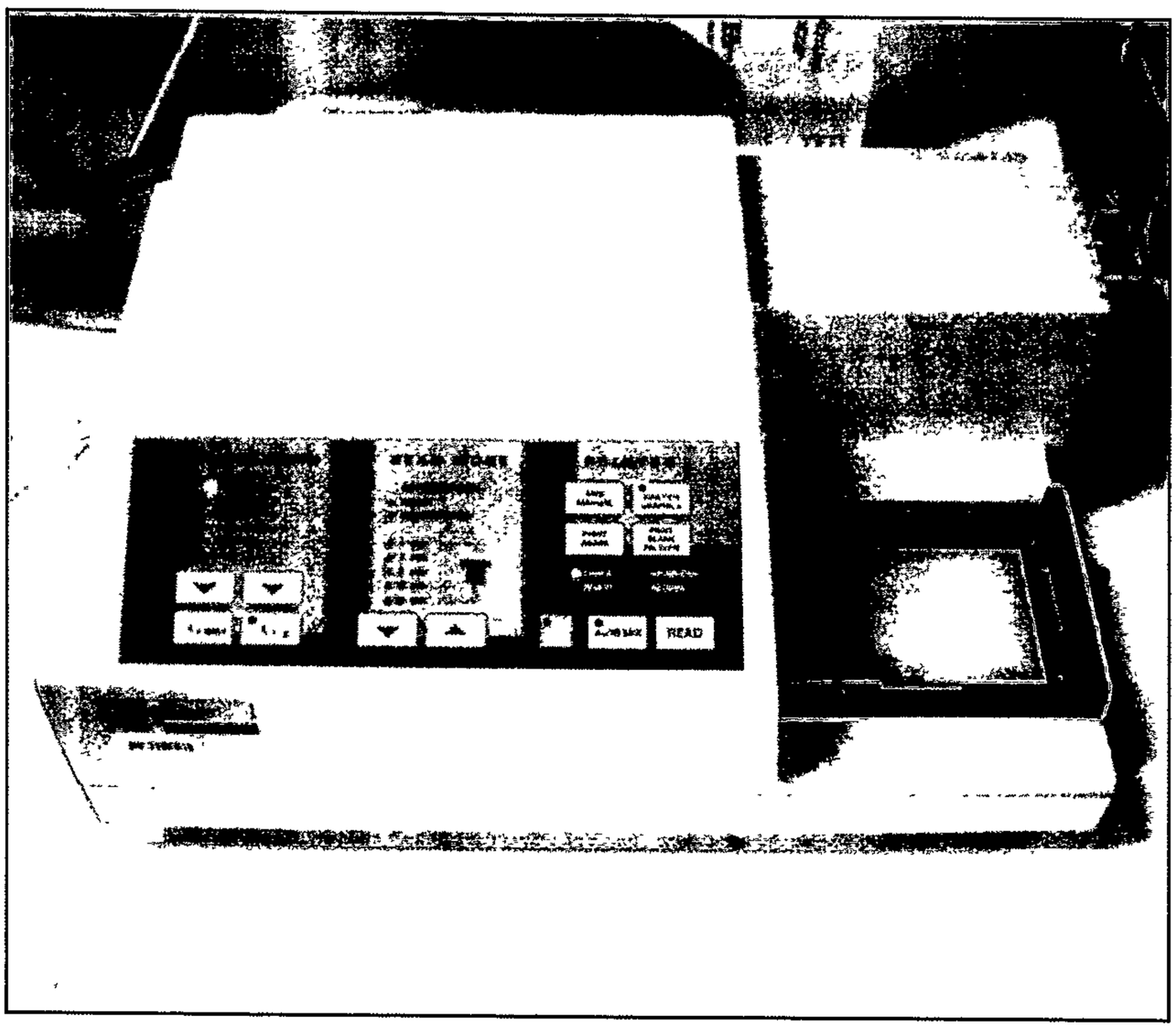
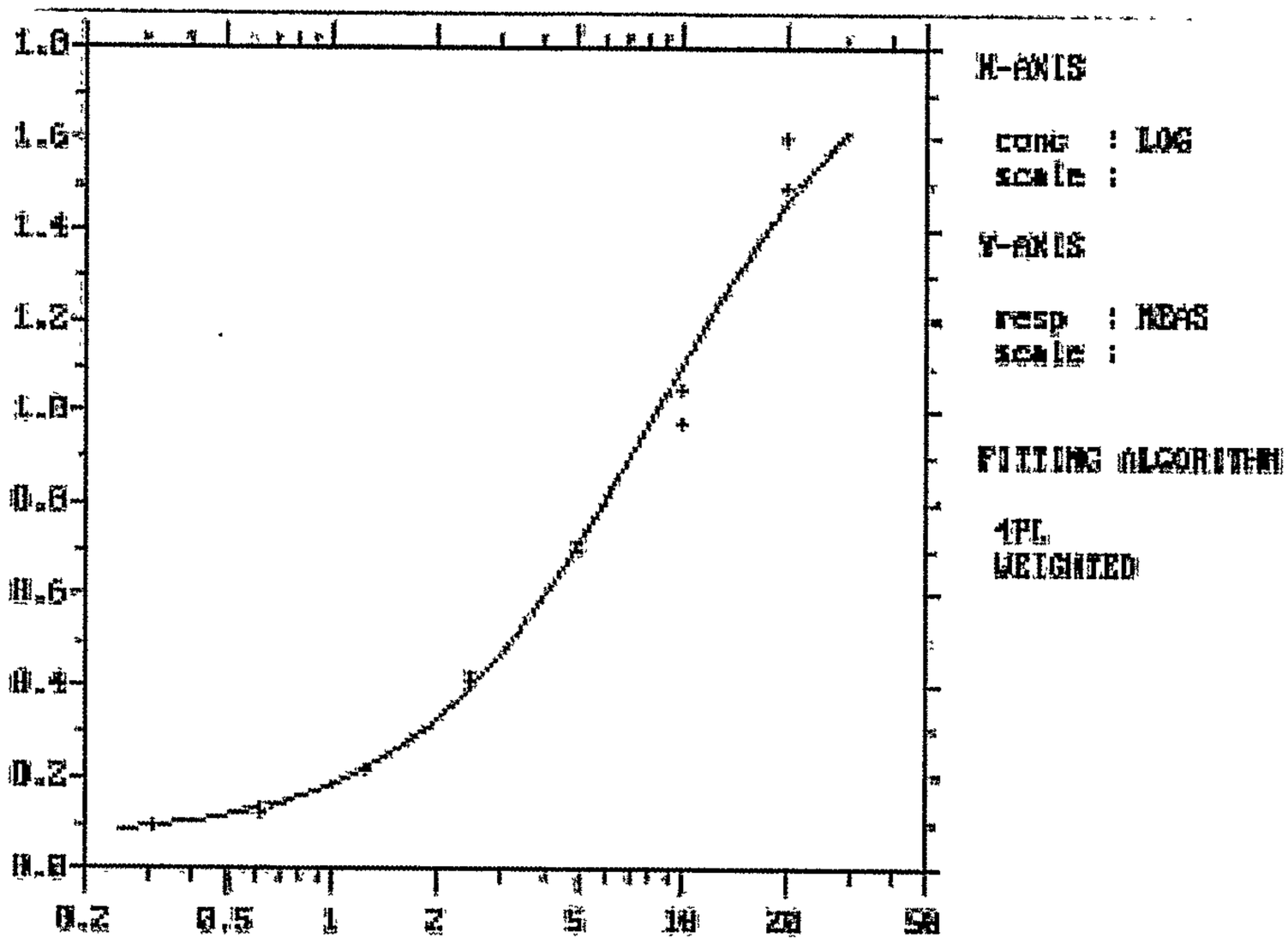


Figure 9 – Plate washing between ELISA reagents



4PL-model : resp = D + (A-D) / [1 + (conc/C)^B]

D= ESTIMATED BLANK = 0.0650
A= ESTIMATED REFER = 1.9169
B= SLOPE FACTOR = -1.265
C= TURNING POINT = 8.2574

BLANK = not coded (0.0650)
REFER = not coded (1.917)

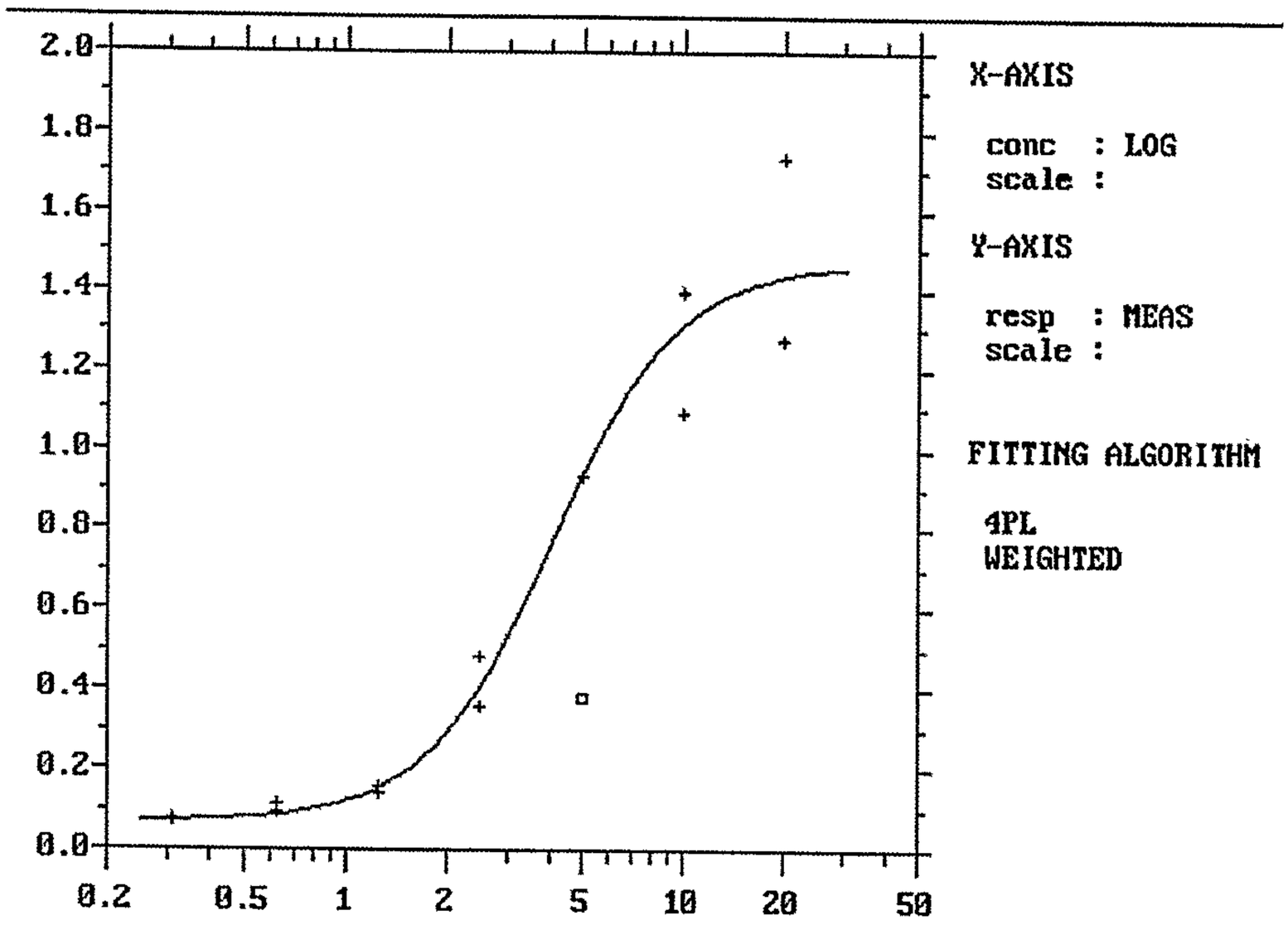
Standard Curve for determination of osteocalcin concentration (first ELISA kit)

ED-20 = 2.269 ED-50 = 5.697 ED-80 = 11.61

X-AXIS (CONCENTRATION) = LOG
Y-AXIS (RESPONSE) = MEAS
FITTING ALGORITHM = 4PL /W

SLOPE = 1.265
INTERCEPT = 2.671
VARIANCE RATIO = 4.083

STD	CONC	CALC.CONC	%DIFF	RESPONSE		%cvRESP	%cvCONC
1.1	0.313	0.313	0.20	0.094			
1.2	0.313	0.313	0.20	0.094			
AVG.	0.313	0.313	0.20	0.094	********	0.02	0.01
2.1	0.625	0.669	7.08	0.139			
2.2	0.625	0.533	-14.8	0.121			
AVG.	0.625	0.602	-3.73	0.130	********	9.79	16.1
3.1	1.250	1.252	0.19	0.221			
3.2	1.250	1.218	-2.56	0.216			
AVG.	1.250	1.235	-1.19	0.219	********	1.62	1.99
4.1	2.500	2.669	6.78	0.423			
4.2	2.500	2.560	2.41	0.408			
AVG.	2.500	2.615	4.59	0.415	********	2.55	2.95
5.1	5.000	5.051	1.03	0.712			
5.2	5.000	4.929	-1.42	0.699			
AVG.	5.000	4.990	-0.20	0.706	********	1.30	1.73
6.1	10.00	8.077	-19.2	0.978			
6.2	10.00	9.151	-8.49	1.051			
AVG.	10.00	8.597	-14.0	1.015	********	5.09	8.81
7.1	20.00	21.57	7.85	1.493			
7.2	20.00	28.74	43.7	1.600			
AVG.	20.00	24.71	23.5	1.546	********	4.89	20.2



4PL-model : resp = D + (A-D) / [1 + (conc/C)^B]

D= ESTIMATED BLANK = 0.0687
A= ESTIMATED REFER = 1.4681
B= SLOPE FACTOR = -2.313
C= TURNING POINT = 1.0817

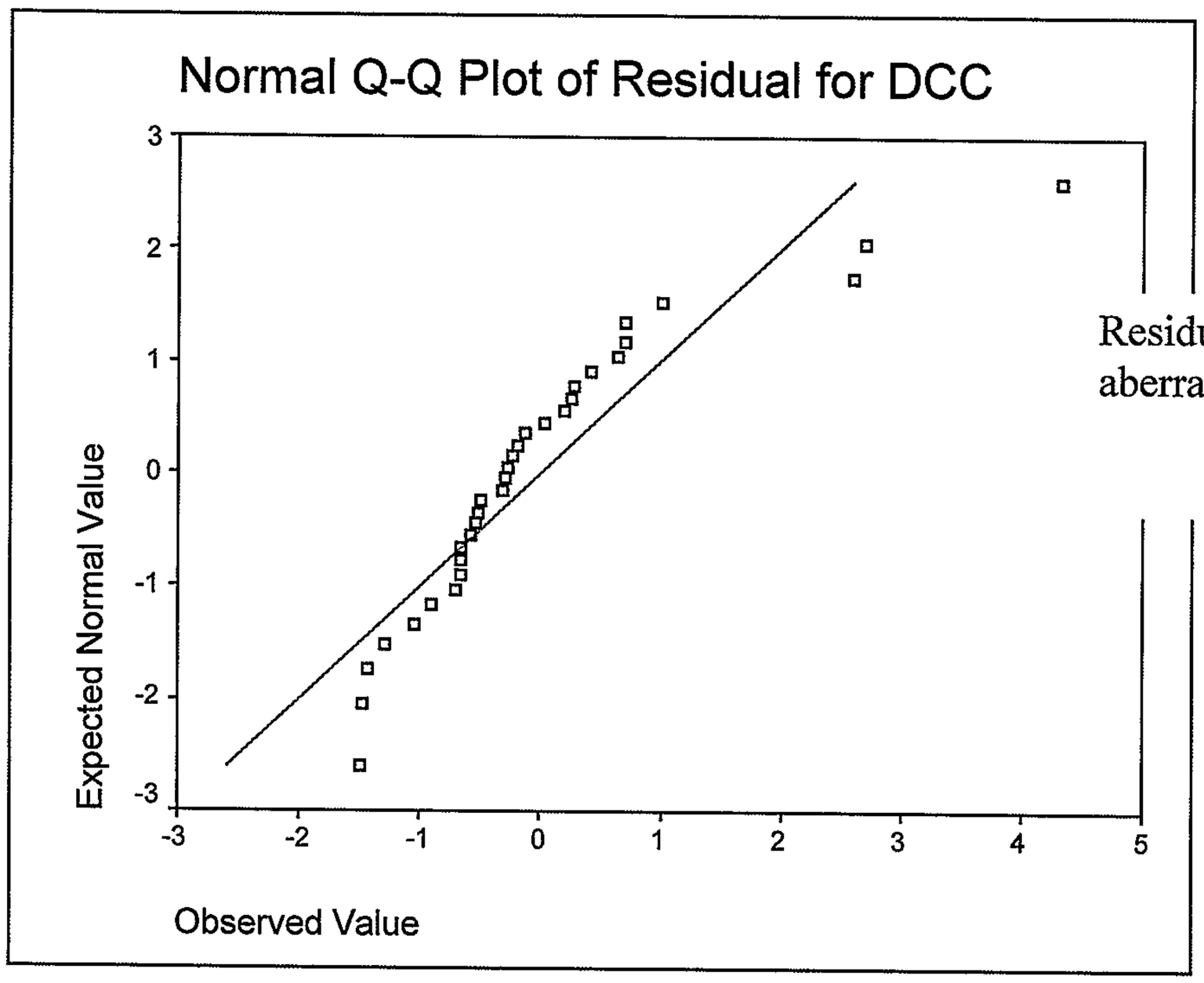
BLANK = not entered (0.0687)
REFER = not entered (1.458)

ED-20 = 0.325 ED-50 = 1.005 ED-80 = 2.068

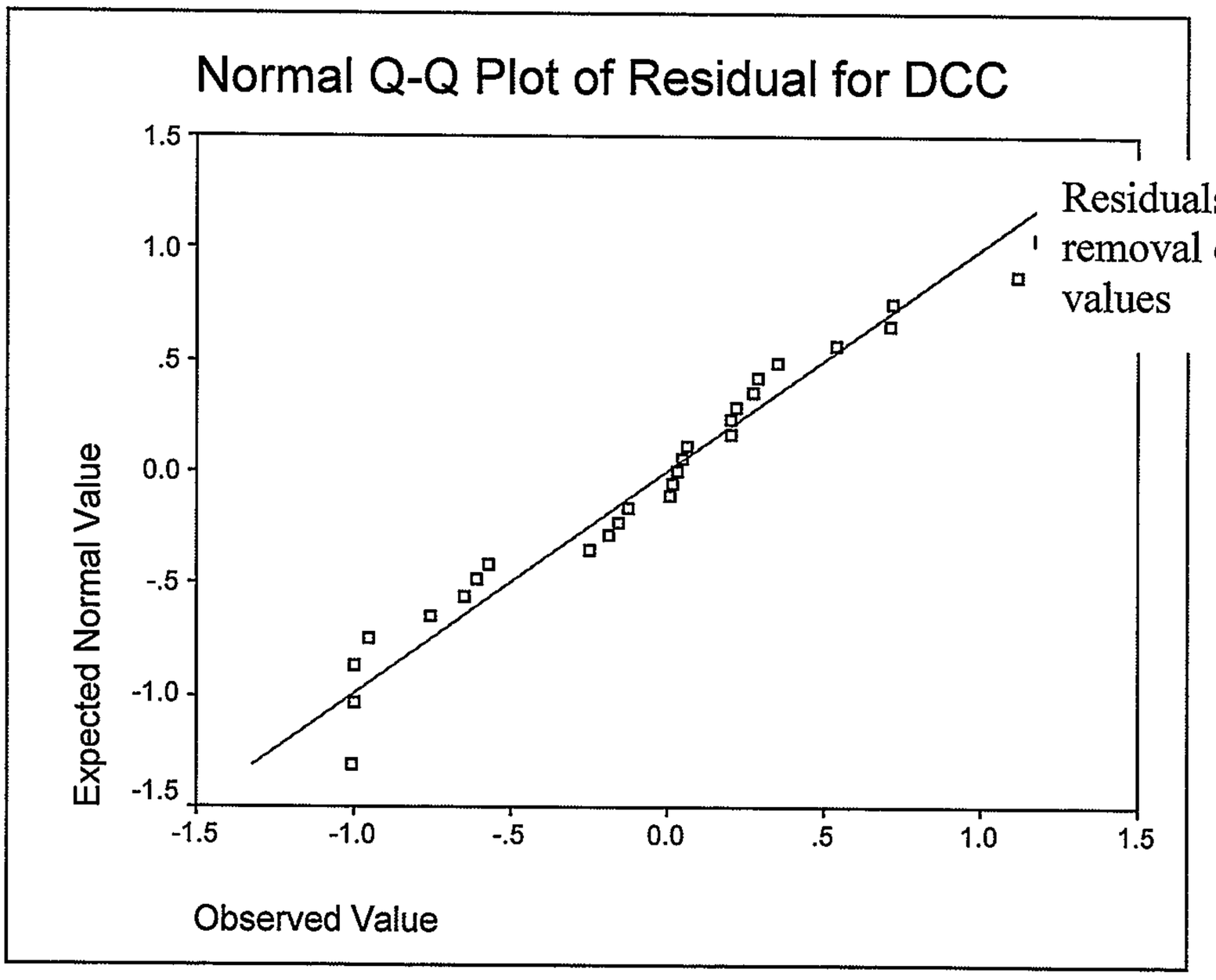
Standard Curve for determination of osteocalcin concentration (second ELISA kit)

X-AXIS (CONCENTRATION)	= LOG			SLOPE	=	2.313	
Y-AXIS (RESPONSE)	= MEAS			INTERCEPT	=	3.254	
FITTING ALGORITHM	= 4PL /W			VARIANCE RATIO	=	0.155	

STD	CONC	CALC.CONC	%DIFF	RESPONSE	%CVRESP	%CVCONC
A.1	0.313	0.366	17.3	0.0740		
A.2	0.313	0.190	-36.5	0.0700		
AVG.	0.313	0.280	-4.61	0.0720	******** 3.93	42.1
B.1	0.625	0.700	12.0	0.092		
B.2	0.625	0.940	50.3	0.114		
AVG.	0.625	0.820	31.2	0.103	******** 15.1	20.7
C.1	1.250	1.175	-6.02	0.143		
C.2	1.250	1.286	2.78	0.159		
AVG.	1.250	1.231	-1.52	0.151	******** 7.49	6.34
D.1	2.500	2.287	-8.53	0.359		
D.2	2.500	2.803	12.1	0.482		
AVG.	2.500	2.547	1.87	0.420	******** 20.7	14.3
E.1	5.000	5.002	0.04	0.930		
E.2	5.000	2.376	-52.5	0.300	REJECTED	
AVG.	5.000	5.002	0.04	0.930	******** 0.00	0.00
F.1	10.00	6.212	-37.8	1.087		
F.2	10.00	13.04	30.4	1.391		
AVG.	10.00	8.260	-17.4	1.239	******** 17.3	54.0
G.1	20.00	9.009837		1.736		
G.2	20.00	8.964	-55.2	1.273		
AVG.	20.00	9.000837	OUT	1.504	******** 21.8	0.00



Residuals plotted with three aberrant values evident



Residuals plotted following removal of the three aberrant values

5. Statistical Analysis

Between-Subjects Factors

		Value Label	N
FL Fluoride	0	no	16
	1	yes	16
SP Springs	0	no	12
	1	yes	20

Tests of Between-Subjects Effects

Dependent Variable: DCC Conc increase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.289[a]	3	4.763	2.728	.063
Intercept	81.089	1	81.089	46.444	.000
FL	.727	1	.727	.416	.524
SP	7.276	1	7.276	4.167	.051
FL * SP	4.903	1	4.903	2.808	.105
Error	48.887	28	1.746		
Total	163.110	32			
Corrected Total	63.175	31			

a. R Squared = .226 (Adjusted R Squared = .143)

Estimated Marginal Means

1. Fluoride

Dependent Variable: DCC Conc increase

Fluoride	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0 no	1.488	.341	.790	2.187
1 yes	1.800	.341	1.101	2.499

2. Springs

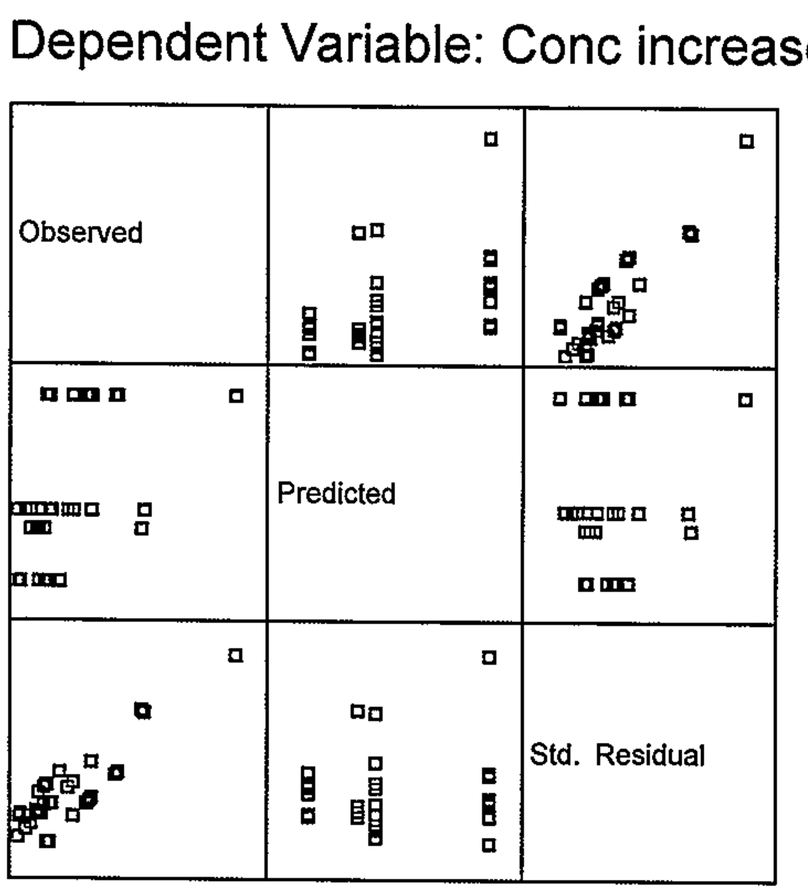
Dependent Variable: DCC Conc increase

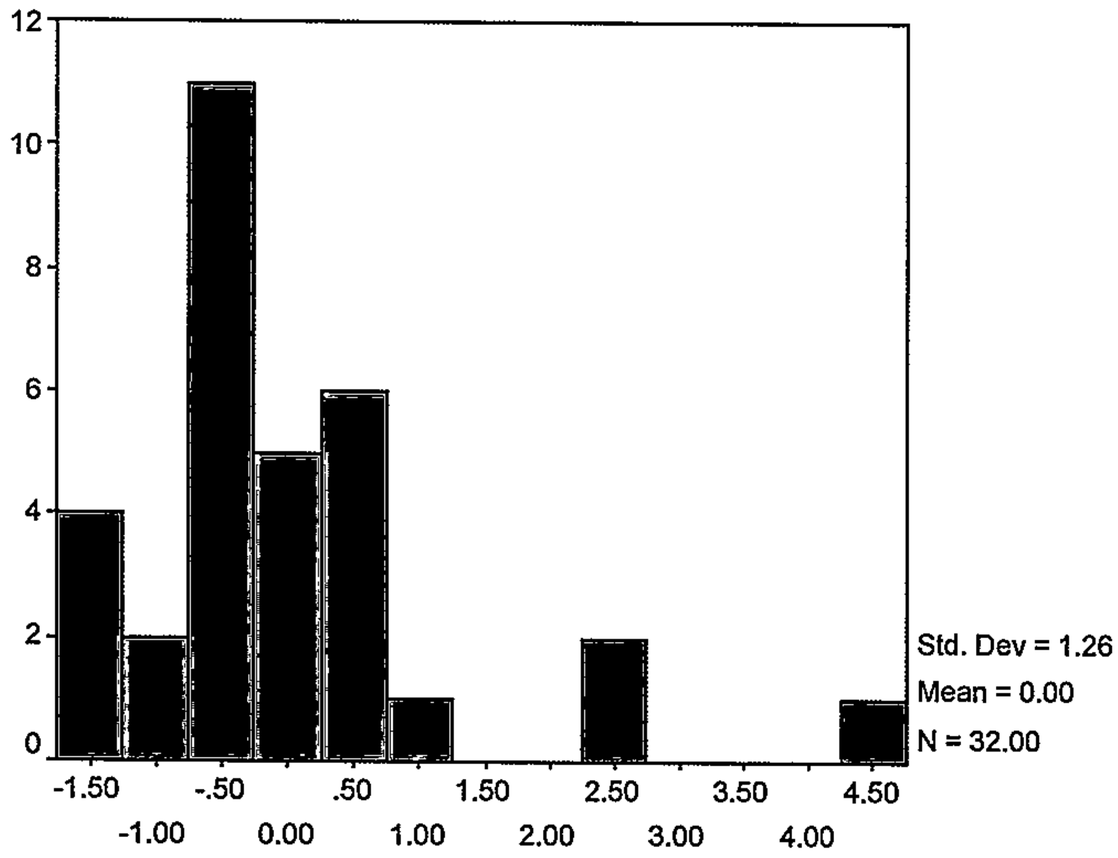
Springs	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0 no	1.152	.381	.370	1.933
1 yes	2.137	.295	1.531	2.742

3. Fluoride * Springs

Dependent Variable: DCC Conc increase

Fluoride	Springs	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0 no	0 no	1.400	.539	.295	2.505
	1 yes	1.577	.418	.721	2.433
1 yes	0 no	.903	.539	-.202	2.008
	1 yes	2.696	.418	1.841	3.552





Statistical Analysis with three aberrant values removed

Between-Subjects Factors

		Value Label	N
FL Fluoride	0	no	14
	1	yes	15
SP Springs	0	no	11
	1	yes	18

Tests of Between-Subjects Effects

Dependent Variable: DCC Conc increase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9.013[a]	3	3.004	6.347	.002
Intercept	47.119	1	47.119	99.534	.000
FL	1.595	1	1.595	3.369	.078
SP	5.132	1	5.132	10.840	.003
FL * SP	1.334	1	1.334	2.818	.106
Error	11.835	25	.473		
Total	79.531	29			
Corrected Total	20.848	28			

a. R Squared = .432 (Adjusted R Squared = .364)

Estimated Marginal Means

1. Fluoride

Dependent Variable: DCC Conc increase

Fluoride	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0 no	1.075	.192	.679	1.470
1 yes	1.559	.181	1.186	1.933

2. Springs

Dependent Variable: DCC Conc increase

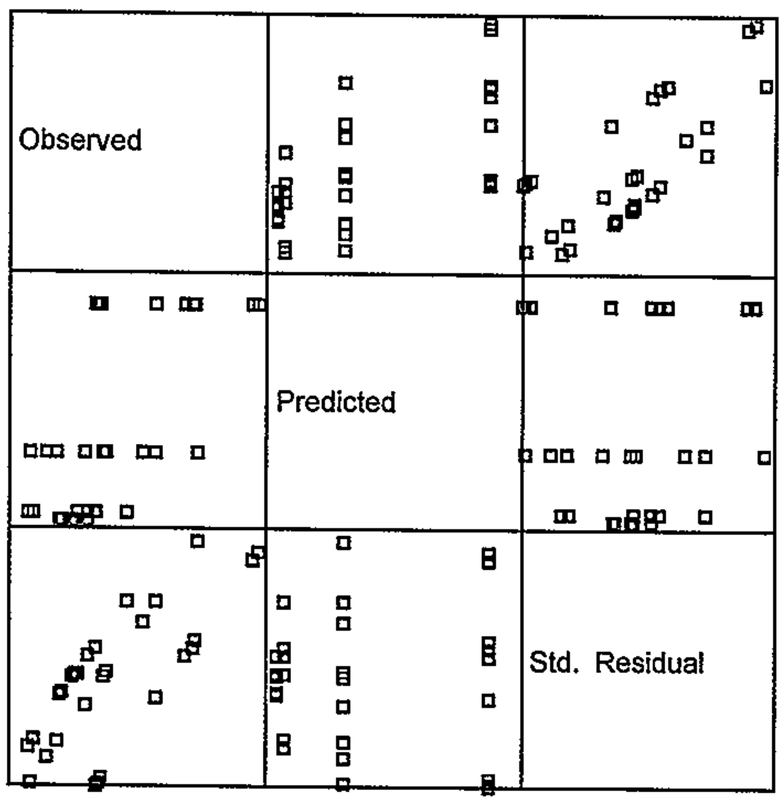
Springs	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0 no	.882	.208	.453	1.311
1 yes	1.751	.162	1.418	2.085

3. Fluoride * Springs

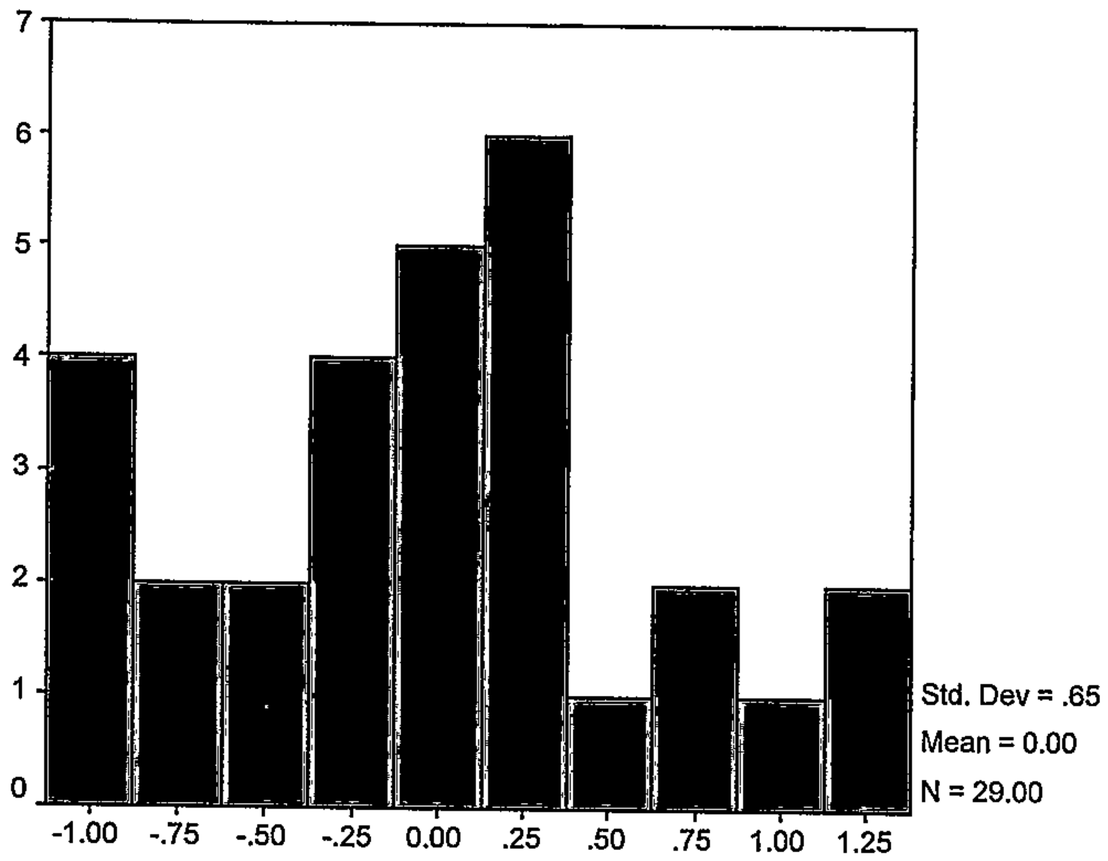
Dependent Variable: DCC Conc increase

Fluoride	Springs	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0 no	0 no	.862	.308	.228	1.495
	1 yes	1.288	.229	.815	1.760
1 yes	0 no	.903	.281	.324	1.482
	1 yes	2.215	.229	1.743	2.688

Dependent Variable: Conc increase



Model: Intercept + FL + SP + FL*SP



Residual for DCC

6. Appendix – ELISA Data

Sequence		Absorb	Conc	%CV	Unit ng/ml	Seq	Absorb	Conc	%CV
A1		0.591	3.976			A10	1.093	6.301	
		0.634	4.343				0.559	3.126	
mean		0.613	4.159	6.2			0.826	4.713	47.6
	1	0.813	6.071			10	1.106	6.433	
		0.797	5.901				1.156	7.001	
		0.805	5.986	2			1.131	6.717	6
A2		0.648	4.466			A9	0.536	3.028	
		0.543	3.584				0.753	4.005	
		0.595	4.025	15.5			0.645	3.517	19.6
	2	0.633	4.334			9	0.937	5.048	
		0.682	4.772				1.168	7.154	
		0.658	4.553	6.8			1.053	6.101	24.4
A3		0.478	3.079			J	0.8	4.244	
		0.494	3.201				0.944	5.095	
		0.486	3.14	2.7			0.872	4.669	12.9
	3	0.615	4.179			J	1.057	5.963	
		0.673	4.689				1.05	5.902	
		0.644	4.434	8.1			1.053	5.933	0.7
A5		0.664	4.608			I	0.745	3.965	
		0.653	4.51				0.964	5.232	
		0.658	4.559	1.5			0.854	4.599	19.5
	5	0.882	6.85			I	1.046	5.867	
		0.634	4.343				1.316	10.135	
		0.758	5.596	31.7			1.181	8.001	37.7
A7		0.625	4.265			C4	0.869	4.626	
		0.655	4.528				0.849	4.511	
		0.64	4.396	4.2			0.859	4.568	1.8
	7	0.981	8.119			C4	0.867	4.614	
		1.043	9.026				1.054	5.937	
		1.012	8.573	7.5			0.961	5.276	17.7
A8		0.538	3.544			C5	1.018	5.635	

		0.728	5.204			1.098	6.351	
		0.633	4.374	26.8		1.058	5.993	8.4
	8	1.043	9.026		C5	0.936	5.041	
		0.389	2.423			1.261	8.698	
		0.716	5.725	81.6		1.099	6.87	37.6
A		0.335	2.041		C6	0.804	4.265	
		0.355	2.182			0.722	3.854	
		0.345	2.112	4.7		0.763	4.06	7.2
A		0.597	4.026		C6	0.83	4.405	
		0.728	5.204			1.005	5.553	
		0.663	4.615	18		0.918	4.969	16.1
B		0.344	2.104		CD	0.601	3.305	
		0.282	1.673			0.724	3.863	
		0.313	1.889	16.1		0.663	3.584	11
B		0.616	4.187		CD	0.667	3.598	
		0.676	4.717			0.798	4.234	
		0.646	4.452	8.4		0.732	3.916	11.5
C		0.48	3.094		CE	1.08	6.175	
		0.558	3.705			1.04	5.816	
		0.519	3.399	12.7		1.06	5.995	4.2
C		0.755	5.47		CE	1.264	8.763	
		0.822	6.168			1.012	5.588	
		0.789	5.819	8.5		1.138	7.175	31.3
D		0.308	1.853		CF	0.692	3.713	
		0.416	2.618			0.846	4.494	
		0.362	2.236	24.2		0.769	4.103	13.5
D		1.032	8.858		CF	0.995	5.457	
		1.083	9.669			1.059	5.981	
		1.057	9.263	6.2		1.027	5.719	6.5
E		0.392	2.445					
		0.434	2.75					
		0.413	2.598	8.3				
E		0.539	3.552					
		0.603	4.077					
		0.571	3.815	9.7				
F		0.423	2.669					

	0.35	2.147	
	0.387	2.408	15.4
F	0.783	5.754	
	0.781	5.734	
	0.782	5.744	0.3
G	0.491	3.178	
	0.531	3.489	
	0.511	3.333	6.6
G	0.663	4.599	
	0.649	4.474	
	0.656	4.537	1.9
H	0.576	3.852	
	0.562	3.737	
	0.569	3.795	2.1
H	0.716	5.089	
	0.857	6.559	
	0.786	5.824	17.8
C1	0.319	1.93	
	0.459	2.936	
	0.389	2.433	29.2
C1	0.9	7.067	
	0.805	5.985	
	0.852	6.526	11.7
C2	0.304	1.826	
	0.318	1.923	
	0.311	1.874	3.7
C2	0.524	3.434	
	0.396	2.474	
	0.46	2.954	23
C3	0.417	2.626	
	0.391	2.438	
	0.404	2.532	5.2
C3	0.509	3.317	
	0.496	3.216	
	0.502	3.266	2.2
CA	0.316	1.909	

		0.296	1.77	
		0.306	1.84	5.3
CA		0.468	3.003	
		0.452	2.883	
		0.46	2.943	2.9
CB		0.313	1.888	
		0.349	2.14	
		0.331	2.014	8.8
CB		0.465	2.981	
		0.456	2.913	
		0.461	2.947	1.6
CC		0.631	4.317	
		0.598	4.035	
		0.614	4.176	4.8
CC		0.589	3.96	
		0.696	4.901	
		0.642	4.43	15
Sample 2				
A4		0.601	3.987	
		0.578	3.785	
		0.589	3.886	3.6
	4	0.627	4.238	
		0.614	4.108	
		0.62	4.173	2.2
A6		0.609	4.068	
		0.636	4.328	
		0.623	4.198	4.4
	6	0.684	4.833	
		0.691	4.917	
		0.688	4.875	1.2

LEGEND

Fluoridated sample	A
Post-mortem	A
Non-fluoridated sample	1
Post-mortem	1
Control (fluoride)	CA
Post-mortem	CA
Control (non-fluoride)	C1
Post-mortem	C1

Conc - Concentration of osteocalcin
CV - Coefficient of variation
Absorb - Absorbance at 450 nm

The pain passes. The beauty remains.

Pierre-Auguste Renoir