The effects of increased intracortical remodeling on microcrack behaviour in compact bone.

AUTHOR(S)

Oran Kennedy, Orlaith Brennan, Peter Mauer, Susan M. Rackard, Fergal O'Brien, David Taylor, Clive Lee

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Corresponding Author: Dr. Oran Kennedy, Ph.D

Corresponding Author's Institution: Royal College of Surgeons in Ireland

First Author: Oran Kennedy, PhD

Order of Authors: Oran Kennedy, PhD; Orlaith Brennan, PhD; Peter Mauer; Susan M Rackard, MVB PhD; Fergal J O'Brien, PhD; David Taylor, PhD DSc; Clive Lee, MD PhD

Abstract: The behaviour of microdamage in bone is related to its microstructural features and thus has an important role in tissue structural properties. However, it is not known how cracks behave in areas of increased intracortical remodeling. More remodeling creates wider variation in the properties of the primary microstructural features of cortical bone, namely osteons. This situation may occur after treatment involving parathyroid hormone or events such as menopause/ovariectomy. High turnover was modelled in this study by using ovariectomy (OVX) to induce surgical menopause in sheep. We hypothesized that osteon age would influence microcrack behaviour during propagation. Five fluorochrome dyes were administered intravenously at different time-points over 12 months post-OVX to label remodeling sites and all animals were then euthanized. Compact bone specimens (2x2x36mm) were harvested from the right metatarsal. Samples were cyclically loaded to failure and then histological analyses were carried out. Cracks were categorized by length into three groups; short (<100mirons), intermediate (100-300microns) and long (>300microns). Numerical crack density (Cr.Dn) of long cracks was greater in controls compared with OVX. Controls also displayed a higher crack surface density (Cr.S.Dn) compared with OVX ($p<0.05$). The behaviour of short cracks did not differ between old and new osteons, but intermediate and long cracks preferentially stopped at newer osteons compared with older ones ($p<0.05$). This mechanism may have an important role in terms of prolonging fatigue life. We conclude that recently formed secondary osteons have

a unique influence on propagating microcracks compared with older osteons. Therefore localised remodeling levels should be considered when studying microcrack behaviour in bone.

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Opposed Reviewers:

Oran Kennedy, PhD
Department of Anatomy
123 St. Stephen's Green, Dublin 2, Ireland
Tel: +353 1 402 2147 Fax: +353 1 402 2355
Email: okennedy@rcsi.ie

Royal College of Surgeons in Ireland
Coláiste Ríoga na Máinlia in Éirinn

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Dear Sir/Madam,

Please find my revisied manuscript attached with this letter. This is my first time to submit an article to your Journal, I hope that everything is in order.

Many Thanks,

Oran Kennedy

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AUTHORS: Oran D. Kennedy,[1,2] Orlaith Brennan,[1,2] Peter Mauer, [1,2] Susan M. Rackard,[3] Fergal J. O'Brien,[1,2] David Taylor,[1] and T. Clive Lee[1,2]

EMAIL: okennedy@rcsi.ie, obrennan1@rcsi.ie, petermauer@rcsi.ie sue.rackard@ucd.ie, fjobrien@rcsi.ie, dtaylor@tcd.ie, tclee@rcsi.ie.

AFFILIATIONS: 1: Trinity Centre for Bioengineering, Department of Mechanical and Manufacturing Engineering, Trinity College Dublin, Dublin 2, Ireland.
Tel: +353 1 8961383 Fax: +353 1 6795554
2: Department of Anatomy, Royal College of Surgeons in Ireland, Dublin 2, Ireland.
Tel: +353 1 4022147 Fax: +353 1 4022355
3: School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Dublin 4, Ireland.
Tel: +353 1 7166056 Fax: +353 1 7166005

Corresponding Author:

Oran Kennedy
Department of Anatomy,
Royal College of Surgeons in Ireland,
Dublin 2, Ireland.
Tel: +353 1 4022147 Fax: +353 1 4022355

ABSTRACT: The behaviour of microdamage in bone is related to its microstructural features and thus has an important role in tissue structural properties. However, it is not known how cracks behave in areas of increased intracortical remodeling. More remodeling creates wider variation in the properties of the primary microstructural features of cortical bone, namely osteons. This situation may occur after treatment involving parathyroid hormone or events such as menopause/ovariectomy. High turnover was modelled in this study by using ovariectomy (OVX) to induce surgical menopause in sheep. We hypothesized that osteon age would influence microcrack behaviour during propagation. Five fluorochrome dyes were administered intravenously at different time-points over 12 months post-OVX to label remodeling sites and all animals were then euthanized. Compact bone specimens (2x2x36mm) were harvested from the right metatarsal. Samples were cyclically loaded to failure and then histological analyses were carried out. Cracks were categorized by length into three groups; short (<100μm), intermediate (100-300μm) and long (>300μm). Numerical crack density (Cr.Dn) of long cracks was greater in controls compared with OVX. Controls also displayed a higher crack surface density (Cr.S.Dn) compared with OVX (p<0.05). The behaviour of short cracks did not differ between old and new osteons, but intermediate and long cracks preferentially stopped at newer osteons compared with older ones (p<0.05). This mechanism may have an important role in terms of prolonging fatigue life. We conclude that recently formed secondary osteons have a unique influence on propagating microcracks compared with older osteons. Therefore localised remodeling levels should be considered when studying microcrack behaviour in bone.

Keywords: Bone turnover; Remodeling; Fatigue; Microcrack; Ovariectomy

Introduction

Traditionally only changes in bone mineral density (BMD), as measured by dual energy x-ray absorptiometry (DEXA), have been considered to be a significant predictor of fracture risk [1]. However, this only accounts for 60-70% of the variation in bone strength, thus some other important factors are not captured by DEXA in the progression of diseases such as osteoporosis [2]. Clinical trials have shown that the fracture risk of a 75 year old woman is 4-7 times greater than that of a 45 year old woman with an identical BMD [3]. It is now widely accepted that bone quality is a combination of parameters which contribute to bone strength, independently of BMD [4, 5]. One important contributor to bone quality is microdamage accumulation and its subsequent behaviour under load.

One form of microdamage which occurs *in vivo* is linear microcracking, caused by the bone being subjected to repetitive sub-maximal loads. These microcracks are normally found in the matrix and are typically 100μm long in the transverse direction [6-11]. The ability of bone to withstand fatigue loading is a function of its ability to resist crack initiation and propagation. These two factors are in turn highly dependent on the microstructural characteristics of the material. The nature of microdamage and its location in the bone matrix plays a role in determining age-related fragility of cortical bone.

The process of damage accumulation in bone under fatigue loading has been extensively investigated in the literature. Early studies analysed fatigue-induced microdamage by

stopping the tests prior to failure to allow for histological analyses of damage which occurred before fracture [12, 13]. Other researchers have studied the process of initiation and propagation of individual microcracks [14]. It was found that the microdamage burden increased rapidly in the early fatigue life of compact bone, the process then stabilized for much of the second phase of the bone's fatigue life, and then increased rapidly again before failure. This demonstrates that bone is able to sustain a significant amount of sub-critical damage before failure. O'Brien et al [15] found that microcrack behaviour on encountering osteons depended on their length.

However, it is still not known how microdamage behaves in areas of increased bone remodeling, such situations may occur after the menopause and in early stage osteoporosis. This situation was modelled in this study by carrying out ovariectomy (OVX) procedures on skeletally mature sheep. It has been shown in a related study on the same animals that OVX increased the amount of intracortical bone remodeling sites, as measured by the numerical density of osteons that had been labeled with fluorochrome dyes [17]. We hypothesize that the age and nature of secondary osteons encountered by cracks during propagation affect their behaviour. It is known that increased bone turnover is a feature of osteoporosis and, although no direct clinical data are available at present on microdamage accumulation in osteoporosis, recent research has shown an association between low BMD and aging in bisphosphonate-treated patients [18]. Thus, the overall aim of this study was to investigate the fatigue properties of bone from control and OVX animals and to characterize the behaviour of fatigue-induced microdamage in relation to new secondary osteons at various stages of development.

Materials and methods

Animal model and experimental procedures

Thirty-eight skeletally mature ewes of mixed breed were randomly divided into an ovariectomy group (OVX; n=19) and a control group (n=19). The precise animal age was not known, however all animals were more than 4 years old. Animals in the OVX group underwent ovariectomy at the beginning of month 0 under Irish Government license. Subsequent veterinary assessment of the animals showed no deleterious effects in the OVX group. Housing, feeding and activity levels were the same for both groups at all times. All animals received an intravenous injection of a fluorochrome dye, via the jugular vein, at the time of surgery and thereafter at 3 month intervals in order to label sites of bone remodeling (Table 1). All animals were euthanized at the end of month 12.

Specimen preparation and fatigue testing

Rectangular parallelepiped beams (2x2x36mm) of cortical bone were obtained from the anterior quadrant of the right metatarsal. All machining was carried out under wet conditions and the specimens were not allowed to dry out at any time. All specimens were stored at -20˚C prior to testing. A horizontally configured, low-force materials testing machine (MTS 250, Tytron, USA) was used to load specimens in 3-point bending fatigue. Load was applied such that compression would be induced towards the endosteal surface and tension towards the periosteal surface. The testing rig had a span of 30mm giving a span/depth ratio of 15. Testing was conducted at a frequency of 3 Hz, with a stress range of 110 MPa and a stress ratio of 0.1. These parameters were chosen based on previous work from our lab [19] and produced tests that would last sufficiently long to demonstrate the 3 phases of fatigue life, but no so long as to be prohibitive. Initial

bending modulus was taken as the maximum slope of the stress-strain curve. Specimens were continually hydrated with saline solution and testing was carried out until outright failure occurred.

Histological analysis

Following failure, specimens were stained *en bloc* with basic fuchsin to label microdamage which had accumulated during the test. An established protocol was used to do this [13, 20]. Transverse histological sections were taken as close as possible to the fracture surface without including any part of it. Sections were then were examined using bright-field microscopy to measure bone area (IX51, Olympus, Hamburg, Germany). Epifluorescence microscopy was carried out to identify microdamage. Linear microcracks were identified using established criteria [8, 15, 21-23].

Microdamage was quantified in terms of crack density (Cr.Dn), which was calculated by dividing the number of cracks by the measured area. Similarly, crack surface density (Cr.S.Dn) was calculated by dividing the combined length of all cracks by the measured area. For the analysis of microdamage interaction with secondary osteons, cracks were categorized into 3 groups by length; short (<100μm), intermediate (100-300μm) and long (>300μm). New osteons were defined as those which had fluorochrome labels incorporated in them, while old osteons were defined as those which had none. New osteons were labeled with one of five fluorochromes, oxytetracycline (administered at the time of OVX), alizarin complexone, calcein, xylenol orange and calcein blue (administered at 3, 6, 9 and 12 months post-OVX). In order to quantify the interaction

between microcracks and old and new osteons the number of microcracks which were arrested at old and each of the differently labeled new osteons was recorded.

Statistical analysis

Variables were expressed as mean ± standard deviation (SD). For statistical analyses, groups were assessed for normal distribution and then compared using a Student's t-test in the case of a two-group comparison. For variables failing the normality test, a nonparametric Mann-Whitney rank sum test was used. One-way ANOVA was used in the case of a multiple-group comparison. All pair-wise multiple comparison procedures were performed using the Holm-Sidak method. SigmaStat 3.0 statistical package (SYSTAT Software Inc, Chicago, USA) was used for all statistical analyses. A p value of <0.05 was considered to be significant.

Results

Animal model and experimental procedures

All animals tolerated the surgery and the administration of fluorochrome dyes without complications. Four animals died during the course of the experiment. Post-mortem examinations revealed that the cause of death in each case was unrelated to experimental intervention and the animals were excluded from the study.

Fatigue testing

The initial bending modulus was reduced in the OVX group compared with controls with values of 17.65 ±2.11 and 20.37 ±7.93 (GPa) (p=0.056). The average number of cycles to failure was 169,557±121,613 and 158,172 ±119,256 in control and OVX groups, respectively the difference between groups was not statistically significant (p= 0.785).

Crack density and crack surface density

Following fatigue testing, Cr.Dn was 3.17±3.02 and 3.52±2.97 (#/mm^2) in control and OVX respectively, the difference was not statistically significant (p=0.736). When the crack length categories were taken into consideration there was, as expected, a decrease in crack number with increasing length (Figure 1). However, Cr.S.Dn was significantly higher in the control group compared to the OVX (p=0.03) (Figure 2). Considering that the control group displayed a higher Cr.S.Dn than the OVX group then it follows, logically, that the control group must contain a large proportion of relatively long cracks In order to quantify this, cracks which were 300μm or longer were measured in the control and OVX groups. The controls contained a significantly higher number of long cracks (>300μm) compared to the OVX group (p=0.003) (Figure 1).

Crack interaction with secondary osteons

Crack interaction with secondary osteons was quantified by considering both groups together; this approach was taken because the issue we wished to address was phenomenological rather than something that resulted from our intervention. Short cracks (<100μm) which encountered secondary osteons were stopped at the cement-line regardless whether the osteon was labeled or unlabeled. When the fluorochrome was taken into consideration, and thus the age of the structure, there was no statistical difference between the numbers of cracks which stopped at any osteon group. However, a non-significant trend towards increased number of short cracks being stopped at more recently formed osteons can be seen (Figure 3a). A higher number of intermediate cracks (100-300μm) were arrested at the boundary of osteons that were formed in the last 6 months of this experiment. This is shown in figure 3b where the number of cracks

stopped at osteons labeled with calcein, xylenol orange and calcein blue are statistically higher compared with those which were unlabeled. Long cracks (>300μm) were also shown to stop more often at recently formed osteons compared with unlabeled. Furthermore, the number of long cracks which stopped at calcein and xylenol orange labeled osteons was greater compared with other earlier labeled osteons (Figure 3c). This effect of new osteons acting as good crack stoppers compared with older osteons is illustrated in Figure 4a where a long crack can be seen to deflect around two unlabeled osteons before being arrested at the boundary of a labeled one. Figure 4b shows an intermediate and a long crack which have propagated towards, and stopped at, a labeled osteon.

Discussion

Understanding the etiology and pathophysiology of osteoporosis is increasingly important as the world's elderly population continues to grow. Although fracture risk is clinically assessed by measuring BMD, the mechanical properties of bone are determined not only by bone mass but also by bone quality [5]. An important parameter of bone quality is the ability of the material to withstand microdamage; the subsequent behaviour of this damage is greatly affected by structure at the microscopic and nanoscopic scales.

It has been shown that secondary osteons in compact bone influence the behaviour of microcracks at different lengths in the bone matrix [15]. This study sought to develop this idea further by considering the effects of variation within the properties of the secondary osteons themselves. Three categories of crack length were defined in this study as this

parameter has been shown to be important in studies of microcrack propagation. The stress intensity of a 300μm crack is higher than that of a 100μm crack, under the same loading conditions, by a factor of approximately 1.7. This is because if the crack length is greater by a factor of 3, then the stress intensity is increased by √3, so this observation is useful in understanding the toughening effect of osteons. We used a fluorochrome labeling system in order to differentiate between new osteons and old ones and thus to assess whether the nature of the osteon influences crack behaviour, in addition to crack length.

This has potential clinical relevance since it is now known that degree of mineralization of bone tissue (DMB) strongly influences the mechanical resistance of bone and also the BMD. This issue becomes relevant in a situation where a course of treatment e.g. parathyroid hormone (PTH), or an event e.g. ovariectomy/menopause, causes an increase in the amount of remodeling events. This could decrease the 'lifespan' of an osteon, because it is more likely to be resorbed away before reaching a state of complete mineralisation [24]. In contrast, anti-resorptive agents (bisphosphonates, estrogens) cause a reduction in the number of remodeling events which can allow more complete secondary mineralisation in pre-existing osteons. Given that the biological determinant of mineralization is the rate of bone turnover, then it follows that understanding how these parameters interact with microdamage, which is also a determinant of bone quality, is an important issue.

The modulus of bending reported here was similar to that measured in a previous study on the sheep metatarsus, using specimens of similar dimensions [25]. Based on a typical slope of an S/N curve in bone [26], the decrease in bending modulus of 13% that we measured would normally result a much greater reduction in N_f. This is because when the groups are normalized with respect to modulus, the group with lower stiffness will effectively see higher stresses. In this study, the groups were normalized by modulus and it was found that the OVX group saw an effective stress of 126.43 MPa compared to the nominal value of 110 MPa in the controls. With this increase, the fatigue life was expected to be reduced by a factor of 11.21. The fact that this reduction was not present, suggests that other microstructural factors must be involved in prolonging the fatigue life in the OVX group.

Cr.Dn was not significantly different in the OVX group compared to controls (p=0.736) However, Cr.S.Dn was significantly higher in the control group compared to the OVX (p=0.003). Furthermore, the number of long cracks (>300μm) was significantly increased in the control group compared to the OVX. We found higher Cr.Dn and Cr.S.Dn values compared with other similar studies in the literature [21], this is primarily because different types of bone were used. We used aged ovine bone which has a high osteon density relative to bovine bone. If we consider bone to be a composite fiber material, with osteons as fibers [27], and fiber-spacing is decreased by increased remodeling activity then this would provide more crack initiation sites in interstitial regions when the bone is cyclically loaded. Secondly, our test specimens were smaller (our cross sectional area

was approximately half the size) and thus would be expected to have a longer fatigue life based on the reduced probability of the specimens containing a critical defect [28].

It is known that the behaviour of microcracks depends on crack length, in other words, the energy that the crack has [15]. It has been shown that a microstructural barrier effect exists in bone whereby crack length influenced microcrack behaviour on encountering an osteon during propagation [15,16], we wished to investigate the effect of osteons at various stages of development, on crack propagation behaviour. In this study, most microcracks were found in interstitial bone which is consistent with the literature [10, 22, 29,30]. We used a fluorochrome labeling system to differentiate between old and new osteons. New osteons were sub-divided into 5 groups based on the fluorochrome that they were labeled with. The behaviour of microcracks in relation to these structures was then characterized.

Short microcracks (<100μm) were the most numerous category of crack found, those that encountered osteons were generally found to stop at the cement-line regardless of whether the osteon was labeled or unlabeled. Statistical analyses showed no differences between any of the 6 categories of osteons. Many of these cracks remained in interstitial bone and had no interaction with osteons, labeled or unlabeled. It is interesting to consider what the mechanism is that causes a short microcrack in interstitial bone to stop without an obvious feature, such as an osteon or a pore, to aid in doing so. This question cannot be answered by the present study, however it has been suggested that the presence

of cracks will serve to redistribute the stresses in the material and thus reduce the stress intensity to a point below the threshold for growth [15]. While there were a large number of short cracks found, mechanically this is not necessarily very detrimental to the material properties of the bone, particularly in comparison to the effect of the propagation of a smaller number of longer cracks.

Intermediate cracks (100-300μm) stopped preferentially at osteons that were labeled with calcein, xylenol orange and calcein blue compared with unlabeled osteons. Osteons that were labeled with any of these three fluorochromes were, at most, 6 months old. A new osteon will have relatively low mineralization compared with an older structure. Lower mineralization may lead to higher localized strains within the osteon, causing newer osteons to behave more like pores in the matrix rather than a functional load bearing volume of material. This effect appears to stop with osteons that are older than 6 months old, as evidenced by the lack of statistical difference between cracks that stop at osteons labeled with oxytetracycline, alizarin complexone and unlabeled ones. These data compare well with the literature where it has been shown that the mineral apposition rate of aged ewes is comparable with that of men and post-menopausal women, which is between 3-6 months [31].

Long cracks (>300μm) were observed to preferentially stop at calcein labeled osteons compared with unlabeled and oxytetracycline, at xylenol orange labeled osteons compared with unlabeled, oxytetracycline and alizarin and finally at calcein blue labeled osteons with compared with unlabeled. As with the intermediate cracks this can be

explained by assuming that newer osteons, containing relatively under-mineralized bone, do not bear much load and so behave like pores in the matrix. Given a favorable combination of stress intensity and damage zones at the crack tip and at the pore, the crack may then propagate towards the osteon and then be blunted by it. This effect has been described in the literature in relation to other materials [32]. There was a significant difference between the number of long cracks that stopped at calcein and xylenol orange labeled osteons compared to other labeled osteons as well as to unlabeled osteons. This may be explained as follows; the longer a crack is the more energy it has thus, a 9 or 12 month old osteon may still be 'young' enough to arrest the growth of an intermediate crack, but not of a longer crack (>300μm). Thus the difference between early labels (0 and 3 months) and later labels (6-12 months) is only apparent when long cracks are considered.

The OVX group, having a lower elastic modulus, would be expected to have a shorter fatigue life, but our results show that this was not the case. We demonstrated that, thanks to increased numbers of under-mineralized osteons, bone with high numbers of recently formed remodeling sites has an increased ability to impede the growth of relatively long cracks, preventing them from growing as easily as they would do in normal bone. The relationship between microstructure and mechanical properties is a complex one: this study has highlighted one rather unexpected consequence of an increased number of compact bone remodeling sites. The material's integrity has been jeopardized, as evidenced by its reduced elastic modulus and a slight increase in the number of fatigue cracks initiated, but this has been compensated by the toughening effect of the new

osteons. Similar effects are well known in other materials, whereby toughness and fatigue resistance can be increased by introducing voids and weak inclusions. However this is a difficult balancing act: if there are too many voids their negative effects will dominate. This study, suggests a reason why early indicators of the osteoporosis, such as increased bone turnover, may be observed a considerable time before the mechanical integrity of the material declines.

In summary, this study examined the effects of increased number of compact bone remodeling sites on fatigue behaviour and on the interaction of microcracks with secondary osteons. High bone turnover resulted in reduced stiffness in the OVX group. While this would normally reduce the fatigue life of a material, that reduction was not observed here. This suggests that some mechanism at the microstructural level may be compensating for the reduced material stiffness. New osteons, recognizable by their fluorochrome labels, were found to be better at stopping intermediate and long propagating cracks outright. More new osteons were present in the OVX group compared with the control, and thus presents a reason for the absence of reduced fatigue life in the OVX group. This study shows that the nature of any obstacles a crack may meet must be considered, as well as the properties of the crack itself, in order to fully understand its behaviour during propagation.

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Figure(s)

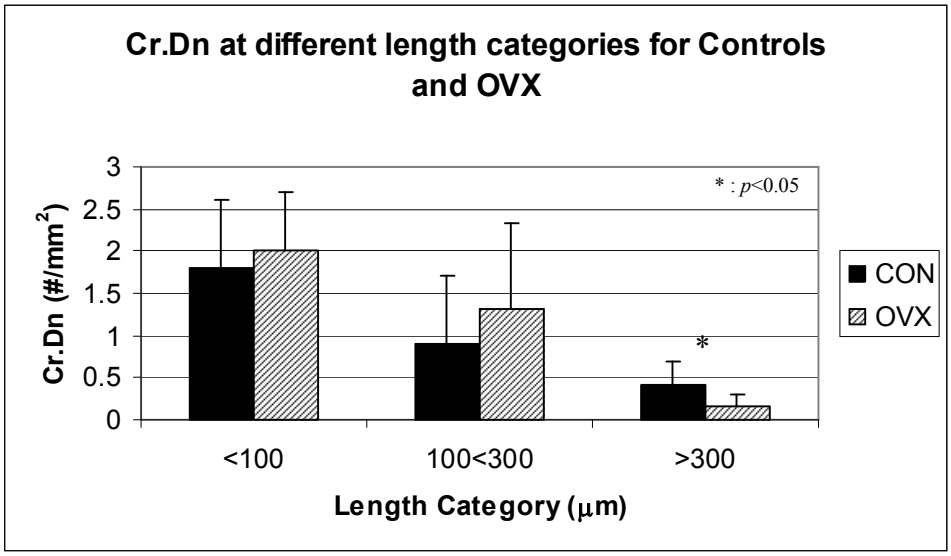

Figure 1: Graph of numerical crack density in control and OVX bone for the 3 different length categories

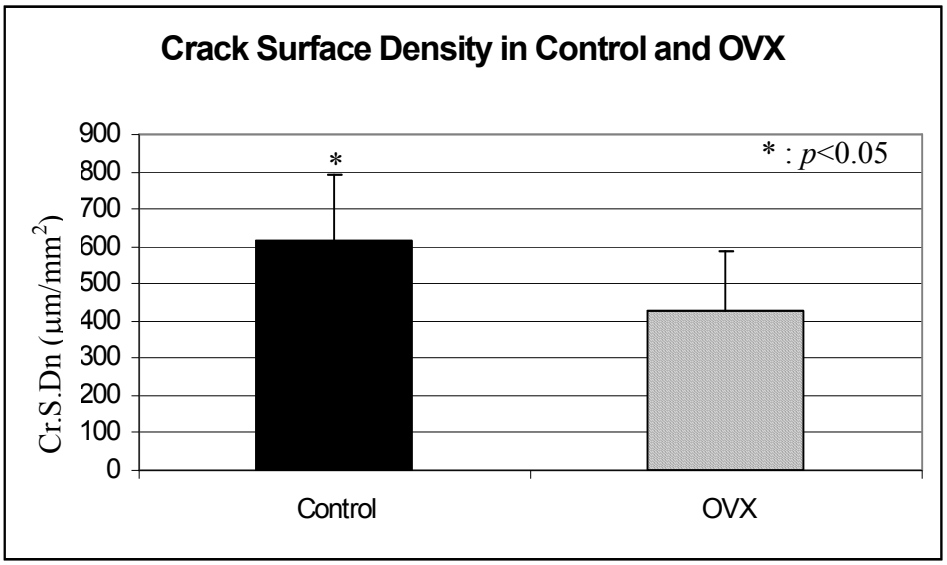

Figure 2: Graph of crack surface density in control and OVX bone samples.

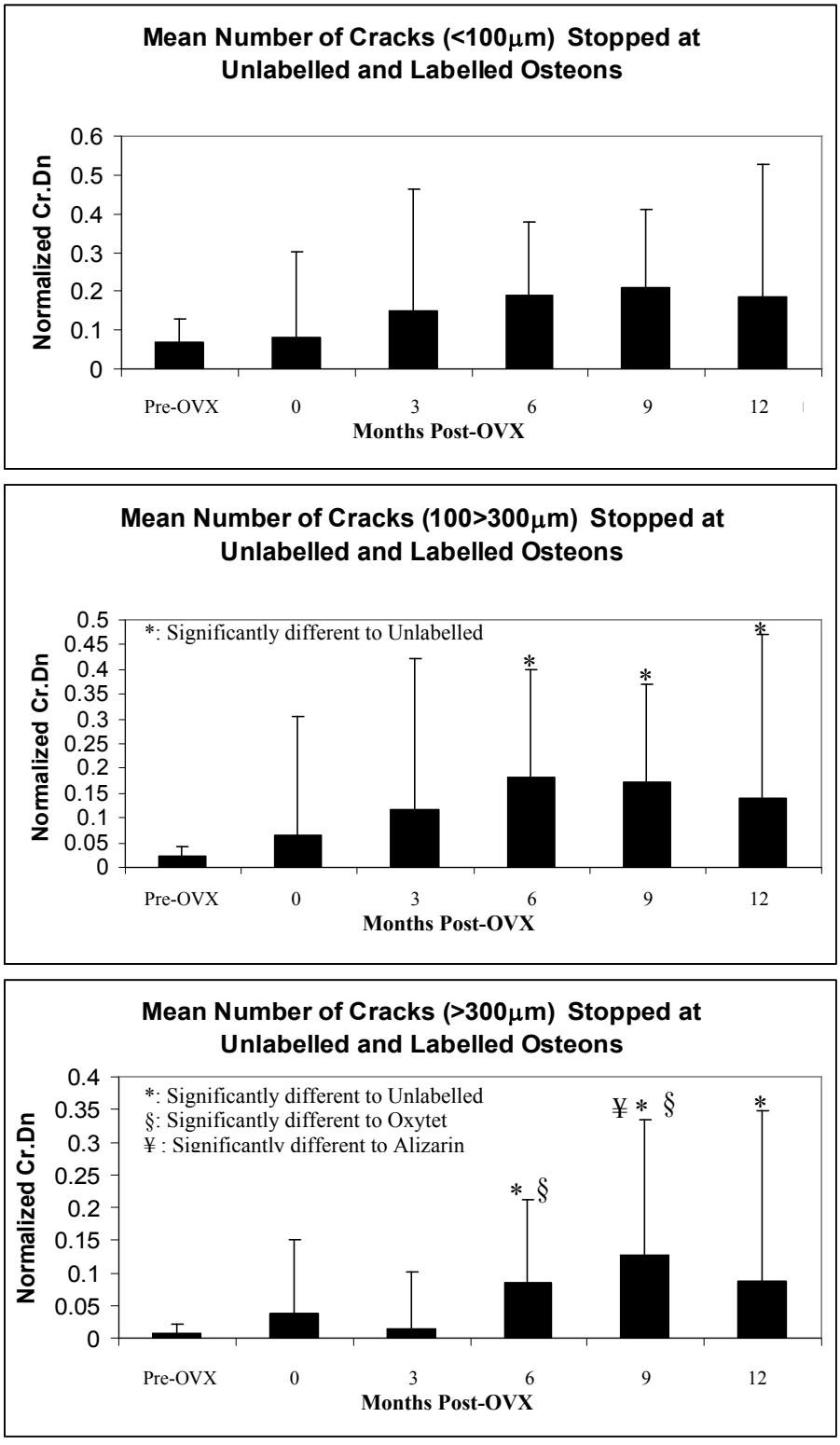

Figure 3: Mean Cr.Dn, of both groups combined, for each category of osteon, normalized by the number of osteons in each case for (a) short cracks (<100μm), (b) intermediate cracks (100<300μm) and (c) long cracks (>300μm).

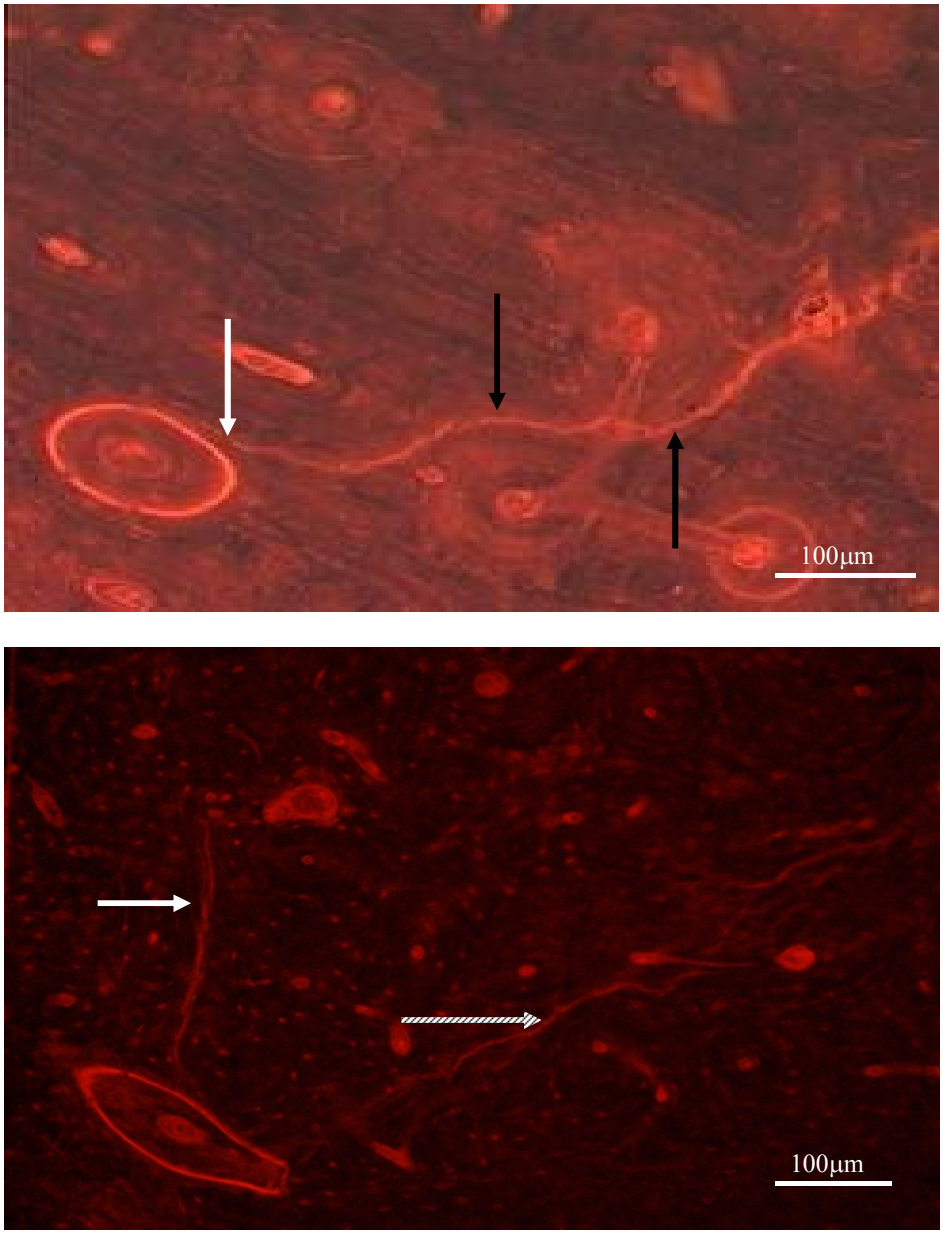

Figure 4: (a) Long microcrack, viewed using green epifluorescence, being deflected around two unlabelled osteons (black arrows) and arresting at a labelled osteon (white arrow) (b) An intermediate (white arrow) and a long (dashed arrow) crack that have propagated towards, and stopped at, a labeled osteon.

Table(s)

Table 1. Details of fluorochrome administration during experimental period

Fluorochrome	Supplier	Months Post Surgery	Dosage IV [mg/kg]
Oxytetracycline	Pfizer	0 (Nov ’03)	50
Alizarin Complexone	Sigma- Aldrich	3 (Feb ’04)	25
Calcein	Sigma- Aldrich	6 (May ’04)	10
Xylenol Orange	Sigma- Aldrich	9 (Aug ’04)	90
Calcein Blue	Sigma- Aldrich	12 (Nov ’04)	30

*** Response to Reviewers**

Reviewers' comments:

Reviewer #1: I am satisfied that the authors have attended to the comments and suggestions made in my review and have improved their manuscript.

Thank you for your help.

Reviewer #2: This draft is a much clearer presentation of what was done, and the additional analyses make for a much more informative paper. A few items should be addressed before publication. Once these are addressed, the paper will make a nice addition to the microdamage literature.

1. The abstract states there was no difference between ovx and control in crack density, but ovx in fact had more >300 um cracks. This should be corrected.

This has been corrected, and the difference has been highlighted in the Abstract.

2. The sentence beginning 'This mechanism may have.' in the abstract should be rewritten to express the point more clearly.

This sentence has been re-written as follows: 'This mechanism may have an important role in terms of prolonging fatigue life...'

3. In the 'statistical analysis' section, you need to state how the 6 group comparisons (in fig 4) were made. There seems to be very large error bars for getting significant differences.

This section has been amended to include the details of the ANOVA and Holm-Sidak procedures that were carried out for these comparisons.

4. Isn't Fig 3 the same as the right-hand side of fig 1? If so, fig 3 should be removed and significance indicated in fig1.

Yes - Figure 3 has been removed and the significance indicated in Figure 1.

5. Fig 4: the group labels would be more informative if you used the times in relation to ovx instead of the dye color. Not clear if these groups contain data from ovx, control, or both; this needs to be explained here and in text.

The labels on this graph have been changed as suggested. These groups contain data from both groups because we wished to address this as a general phenomenon as opposed to something which resulted from our intervention. This point has now been clarified in the caption and in the text.