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Case Report

Variability of *in vivo* linear microcrack accumulation in the cortex of elderly human ribs

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ABSTRACT

Excessive accumulation of microdamage in the skeleton *in vivo* is believed to contribute to fragility and risk of fracture, particularly in the elderly. Current knowledge of how much *in vivo* damage accrual varies between individuals, if at all, is lacking. In this study, paired sixth ribs from five male and five female elderly individuals (76–92 years, mean age = 84.7 years) were examined using *en bloc* staining and fluorescent microcopy to quantify linear microcracks present at the time of death (*i.e. in vivo* microdamage). Crack number, crack length, crack density, and crack surface density were measured for each complete cross-section, with densities calculated using the variable of bone area (which accounts for the influence of porosity on the cortex, unlike the more frequently used cortical area), and analyzed using a two-way mixed model analysis of variance. Results indicate that while microcracks between individuals differ significantly, differences between the left and right corresponding pairs within individuals and the pleural and cutaneous cortices within each rib did not. These results suggest that systemic influences, such as differential metabolic activity, affect the accumulation of linear microcracks. Furthermore, variation in remodeling rates between individuals may be a major factor contributing to differential fracture risk in the elderly. Future work should expand to include a wider age range to examine differences in *in vivo* microdamage accumulation across the lifespan, as well as considering the influence of bisphosphonates on microdamage accumulation in the context of compromised remodeling rates in the elderly.

1. Introduction

Microdamage in the skeleton forms under both normal and aberrant loading conditions, with accumulation of microdamage resulting in stress fractures and contributing to increased fragility and fracture risk (Frost, 1960). In young, healthy bone that can effectively remodel (*i.e.* repair) quickly, microdamage is of minimal concern (Burr, 2011). However, in the aging population, where activation frequency and thus the entire remodeling process slows with increasing age, microdamage formation can outpace repair and lead to compromised bone quality (Diab et al., 2005). Additionally, the use of bisphosphonate drugs to treat osteoporosis further impedes repair processes, which research suggests leads to further increased microdamage accumulation (Pazianas et al., 2014; Allen and Burr, 2011). Increased longevity in modern populations has seen a corresponding rise in fracture induced morbidity and mortality, particularly in the context of the thorax, where rib fractures in the elderly often have poor recovery outcomes (Sirmali et al., 2003). As such, studying patterns of microdamage variation is essential to unraveling microdamage's contribution to fracture risk and bone health.

The rib serves as an ideal location for such a study. Unlike the long bones of the appendicular skeleton that undergo a wide range of dynamic loads, mechanical loading in human ribs is likely primarily cyclic in nature due to the regular demands of respiration (Agnew and Stout, 2012; Kondo et al., 2000). Additionally, the rib is highly susceptible to metabolic and physiological changes (Frost, 1969), making it a worthy location to examine the effects of slowed remodeling and decreased activation frequency with increased age.

The objective of this study is to identify variability in *in vivo* microdamage accumulation in the ribs between and within elderly individuals. Such variation could elucidate a potential mechanism of differential fragility. Using *en bloc* staining and fluorescent microscopy techniques, we specifically considered whether significant differences in microcrack accumulation exist (1) between individuals, (2) between left and right bilateral ribs within individuals, and (3) between the pleural and cutaneous cortices within a rib.

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2. Materials and methods

Ribs from post-mortem human subjects (PMHS) received at The Ohio State University's Whole Body Donation Program were used for this research. The sample includes five males and five females aged from 76 to 92 years (mean = 84.7 years). The presence of preexisting thoracic trauma was the only exclusion criterion, therefore the sample utilized is random and assumed to be representative of a "typical" elderly population.

Bilateral sixth ribs were dissected, complete from head to sternal end. All soft tissue, including the periosteum, was carefully removed and two-centimeter transverse segments were cut at 50% of the rib length along its curvature with a Dremel grinding disc. Undecalcified segments were rinsed of marrow and stained *en bloc* using 1% basic fuchsin hydrochloride (JT Baker) in a series of graded ethanol solutions (70, 80, 90, and 100%), a protocol derived from Burr and Condon (pers. comm). This method allows staining of microcracks which existed *in vivo* and excludes those that may be processing artifacts (Burr and Stafford, 1990). Stained bone segments were subsequently embedded in methyl methacrylate and two transverse sections (~80 μm) per rib at the 50% location were sectioned and mounted with Eukitt mounting medium.

Utilizing an Olympus BX51 microscope, overlapping bright field images were taken at 100× magnification across the entire rib cross-section with a Diagnostic Instruments™ digital camera to form a composite cross-sectional image of each rib section. Cortical Area (Ct.Ar) and Porosity Area (Po.Ar) were manually traced on the composite with SPOT™ Advanced imaging software and a digital tablet, with verification through live microscopy. Porosity was defined as any vascular or resorption spaces within the cortex, excluding canaliculi and osteocytic lacunae, and was subtracted from the Ct.Ar to create a Bone Area (B.Ar) variable that represents only the absolute amount of cortical bone within a transverse section.

The MomentMacro plug-in (Ruff, no date) for ImageJ software (NIH) was utilized to define the rib centroid and principle bending axes, after which the primary axis was referenced to systematically separate the pleural from the cutaneous cortex for further data analysis (see (Agnew and Stout, 2012)).

All microcracks present in the cortical bone of each rib section were identified at 400× magnification using a FITC filter and epifluorescent light (Fig. 1). The position of each was marked on the parent composite image to ensure every crack was located and none were counted twice. Microcrack variables were quantified using Microsuite digital software for each of the two sections and an average was subsequently used. Variables were defined according to Burr and colleagues (Burr et al., 1998), with the exception of the use of B.Ar instead of Ct.Ar to calculate crack densities. Variables are:

1) Crack Number (Cr.N); numerical count of all linear microcracks
2) Crack Length (Cr.Le); mean length of all linear microcracks
3) Crack Density (Cr.Dn); calculated as Cr.N/B.Ar
4) Crack Surface Density (Cr.S.Dn); calculated as Cr.N * Cr.Le/B.Ar

A natural log transformation resulted in normally distributed data, for which subject and side were analyzed in a two-way mixed model per cortex using analysis of variance (ANOVA). A paired *t*-test was then used to further assess differences between cortices. Significance was set at 0.05 for all statistical tests.

3. Results

Table 1 provides subject information and subject-level means for each variable. Cr.Le, Cr.Dn, and Cr.S.Dn were found to vary significantly between subjects for each cortex ($p < 0.05$), while no differences were identified between right and left side paired ribs ($p > 0.4$; Table 2). Furthermore, no significant difference were found in Cr.Le, Cr.Dn, or Cr.S.Dn between pleural and cutaneous cortices (paired *t*-test results, $p = 0.88$, $p = 0.19$, and $p = 0.16$, respectively). However, in all instances the cutaneous cortex values were higher than those of the pleural cortex. Cr.Le values for the cutaneous cortex are only slightly greater than those of the pleural cortex (39.48 and 39.26, respectively), while the difference in crack densities was greater. Mean Cr.Dn was 37.96 (±16.36) in the cutaneous cortex and 31.93 (±11.72) in the pleural, while mean Cr.S.Dn was 1536 (±742) in the cutaneous and 1265 (±565) in the pleural cortex.

4. Discussion

This study presents preliminary evidence for variation in microcrack accumulation in a sample of sixth rib pairs from 10 elderly individuals. The results show significant differences in the presence of microcracks in size and areal density measures between individuals. Under the assumption that similar loading conditions are present for sixth ribs of all individuals, these inter-subject differences are likely attributable to differential crack initiation/propagation or related differences in repair rates (*i.e.*, bone turnover). Furthermore, the lack of significant results when comparing paired ribs within an individual supports the notion that systemic factors play a role in the accumulation of skeletal microdamage.

While some researchers have quantified *in vivo* microdamage in human long bones (Schaffler et al., 1995; Norman and Wang, 1997; Zioupos, 2001), to our knowledge, no direct attempt to quantitatively assess *in vivo* measures of linear microcracks in human ribs has been made. Of the few studies that do report *in vivo* microcrack data for the ribs, though they do so incidentally, most have found much lower amounts of linear microcracks, as well as shorter microcracks, than those presented here (Frost, 1960; Burr and Stafford, 1990). The higher values reported here are attributed in part to increased age of the

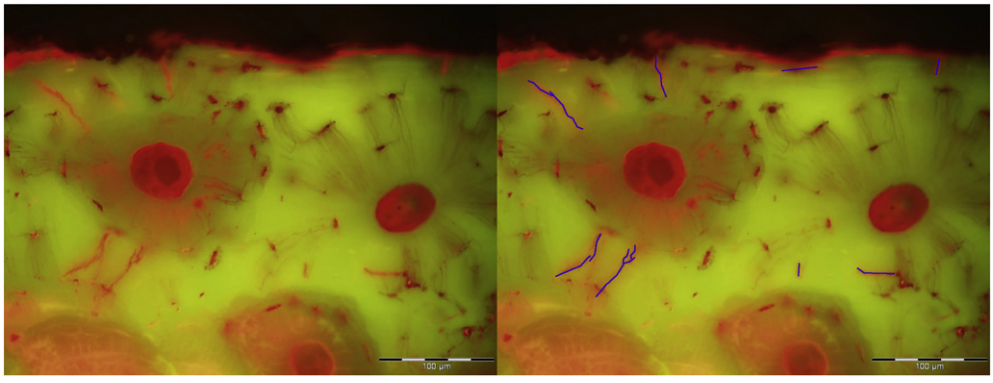

Fig. 1. Example of Linear Microcracks. Representative Field of View (FoV) from a human rib, stained with basic fuchsin and imaged at 400× total magnification under fluorescent light with a FITC filter. For this FoV, the raw image (left) is compared to the same image that includes the observed and measured linear microcracks (right). Scale bar = 100 μm.

Table 1
Summary data per individual[a].

Subject	Age yrs	Sex	B.Ar mm^2	Cr.N #	Cr.Le μm	Cr.Dn # mm^{-2}	Cr.S.Dn μm mm^{-2}
A	88	M	16.10	734.5	48.018	45.598	2189.524
B	92	F	11.25	366.5	36.414	32.559	1185.594
C	77	F	13.86	696.5	39.248	50.229	1971.414
D	76	M	14.72	370	40.618	25.131	1020.773
E	91	F	12.24	282.25	36.375	23.041	838.127
F	83	M	34.04	1829	44.357	53.715	2382.660
G	90	M	23.40	917.25	43.153	39.192	1691.218
H	82	M	19.24	545.25	37.705	28.327	1068.070
I	88	F	13.07	203.5	39.098	15.568	608.679
J	80	F	16.71	564	32.753	33.746	1105.259
Mean	84.7	–	17.46	650.87	39.774	34.711	1406.132

[a] Data presented are means of right and left 6th ribs per subject.

sample (mean age of 84.7 years) and the high magnification (400×) used for this study. The elderly age of our sample increases the likelihood of compromised repair mechanisms, potentially leading to increased microcrack accumulation. Additionally, higher magnification improves visual resolution. What appears as a long, singular microcrack under low magnification, often under high magnification reveals itself to be multiple, shorter cracks.

Another factor that likely influenced our results is the use of B.Ar rather than Ct.Ar when calculating areal density values. B.Ar is smaller than Ct.Ar, measuring the true bone in a cross-section by excluding the surface area of all porous spaces. A paired *t*-test between these variables for this sample showed the difference to be significant ($p < 0.01$), so it is sensible that using B.Ar to calculate density measures will result in inflated numbers when compared to previously published studies. Prior work has suggested utilizing B.Ar to calculate crack densities as it is a more biologically correct measure of altered bone quality, quantified in the context of Po.Ar, which has also shown significant variation between individuals and contributions to differential fragility (Agnew and Stout, 2012; Dominguez et al., 2016). Additionally, the lack of validated descriptions and criteria for counting and measuring microcracks may also contribute to quantitative differences between this and previous studies since there are currently no established standards in existence.

Lastly, we note that linear microcracks are neither the only type of microdamage to form in bone, nor the most prevalent. Other microdamage types, such as diffuse damage that can itself lead to linear microcracks, comprise a larger percentage of bone microdamage, but cannot be detected with light microscopy, requiring instead more powerful methods such as confocal analysis (Skedros et al., 2011). Despite the limitations imposed by considering only one type of microdamage, the results presented here are illuminating. Diffuse damage, another common form of microdamage, tends to occur at lower magnitudes and under tensile loads, rather than linear microcracks, which usually derive from compression and shear loading modes (Diab and Vashishth, 2005; Diab and Vashishth, 2007). As such, linear microcracks are thought more detrimental to bone than other damage types, resulting in compromised material properties and a greater propensity to fracture (Burr et al., 1998). Furthermore, examining the spatial distribution and differences in crack length may help inform our understanding of strain mode and/or magnitude experienced in human ribs (Boyce et al., 1998). The specific biomechanical loading environment of human ribs associated with breathing has yet to be established. Thus, studying linear microcracks can contribute to studies of mechanical adaptation and fracture etiology, though future work should also use confocal microscopy or similarly advanced techniques to fully assess all forms of microdamage in the human rib.

This study indicates that significant variability in microdamage accumulation between elderly individuals could contribute to differential fracture risk and merits further investigation, including expanding the study to include a wider age range to gauge the occurrence of *in vivo* microdamage across the lifespan. As a component of bone quality, a multi-faceted concept that considers the structural and material properties of bone to define bone health, understanding the role of microdamage and its developmental and repair mechanisms in bone holds great promise for improving our understanding of fracture risk (Seeman and Delmas, 2006). Illustrating this point, the subject in this study with the greatest amount of bone (Subject F), also has the highest Cr.Dn (Table 1), supporting the notion that future research should further investigate the role of bisphosphonates in preserving bone area/volume by suppressing remodeling and the potential impact of the resulting increased microdamage on rib fragility in humans. Additionally, since rib pairs were found to vary by subject but not side, future work may use corresponding bilateral pairs for experimental studies directly measuring bone strength, maintaining one side as a control.

Table 2
ANOVA results for microcrack variables by cortex[a].

		Cutaneous		Pleural	
	DF	*F-stat*	*p-value*	*F-stat*	*p-value*
Crack length (Cr.Le)					
Side	1	0.310	0.676	0.021	0.909
Subject	9	8.884	**0.001**	4.804	**0.014**
Interaction	9	0.668	0.721	1.458	0.291
Crack density (Cr.Dn)					
Side	1	0.166	0.753	1.457	0.440
Subject	9	4.443	**0.018**	3.549	**0.036**
Interaction	9	2.693	0.078	1.655	0.232
Crack surface density (Cr.S.Dn)					
Side	1	0.058	0.849	1.114	0.482
Subject	9	4.694	**0.015**	4.660	**0.015**
Interaction	9	4.086	**0.023**	2.415	0.102

[a] Intra-individual variation is expressed as variation between each source "side" to assess directional differences. Inter-individual variation is expressed as variation between each source "subject." The "interaction" refers to the effect of one independent source variable (*i.e.*, side or subject) being influenced by the other. Bolded p-values indicate statistical significance at the $\alpha < 0.05$ level.

Author contributions

A. Agnew designed the study and performed all data collection. V. Dominguez prepared the draft manuscript and provided interpretation of results. P. Sciulli was responsible for statistical analysis of the data. S. Stout provided crucial input on study design, methods, and interpretation. All authors contributed intellectual content and approved the final version of the paper. All authors agree to be held accountable for the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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