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Methodological approach for the detection of both microdamage and fluorochrome labels in ewe bone and human trabecular bone

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Abstract The purpose of this study was to adapt various staining methods for the detection of microdamage in human bone, while preserving tetracycline labels. We describe two staining methods using calcein green and xylenol orange, first developed in ewe bone samples and validated in human trabecular bone samples. In ewe bones, we found that calcein green at 0.5 mM concentration diluted in 100% ethanol as well as xylenol orange at 5 mM were the most adequate fluorochromes both to detect microdamage and preserve the double tetracycline labeling. These results were verified in human trabecular bone (iliac crest for the tetracycline label, and vertebral bone for the double labeling). Results obtained in human bone samples were identical to those in ewes, so this combination of fluorochromes is now used in our laboratory.

Keywords Microdamage · Fluorescent labels · Ewe iliac crest · Human trabecular bone

Introduction

Loading of bone induces in vivo linear and diffuse microdamage [1–10]. The propensity of bone to form linear and diffuse damage varies with age and the two forms of

microdamage have different effects on the mechanical properties of bone and influence skeletal fragility [11–14]. Microdamage has been largely studied in compact bone [13, 15–22] but less in human trabecular bone [5, 23–28]. To date, two main standard methods to stain bone for microdamage detection have been described: (1) the basic fuchsin bulk staining used to detect microdamage generated in vivo [2, 6, 19, 25, 29, 30], and (2) the use of fluorescent chelating agents [16, 17, 19, 31, 32]. In addition to distinguishing fully-stained microcracks generated in vivo from partially-stained or artifactual-stained cracks due to cutting and/or machining, one of the challenges is to identify and quantify microdamage in human trabecular bone without washing previous labels used for histomorphometry and qualitative bone histology.

The aims of this study were to develop a methodological approach, based on previous work [2, 3, 6, 16, 17, 19, 29, 31], to accurately identify microdamage in trabecular bone without erasing fluorescent labels such as tetracycline. This approach has also made it possible to distinguish pre-existing true from artifactual microdamage and damage from mechanical testing. We describe here a double labeling method using two different fluorochromes that was developed on ewe bone samples and then validated on human trabecular bone samples.

Materials and methods

The methods were developed in ewe bone and then applied to human trabecular bone.

Preparation of ewe bone

Fifty transiliac bone biopsies were obtained after tetracycline labeling from ewes under local anesthesia, with a

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Fig. 1 Preparation of transiliac bone biopsies from ewes

7.5-mm internal diameter trephine, 2 cm behind the anterosuperior iliac spine and 2 cm below the iliac crest edge (Fig. 1) [33]. To rule out artifactual microdamage, bone samples needed to be stained before and after cutting the sections. Biopsies were then separated into two parts. One half served as a control to observe double labeling on unstained sections, and the other half served for the fluorochrome tests.

To quantify the correct concentration, dilution and time limit for detecting both microdamage and labels, 34 half-biopsies were bulk-stained separately at room temperature with the following fluorochromes (Table 1; Fig. 2): basic fuchsin¹ (BF), $n = 1$; fluorescein,² $n = 12$; calcein blue,³ $n = 4$; alizarin complexone,⁴ $n = 2$; calcein green,⁵ $n = 6$; and xylenol orange,⁶ $n = 9$. All tests were performed either in ethanol or a water-based solution and different concentrations and duration of bulk staining were tested for each fluorochrome (Table 1). To verify that fluorochrome staining did not alter classical histological stains, a few supplementary sections were cut for Goldner's trichrome, Solochrome cyanin R, May–Grünwald–Giemsa and Toluidine blue [34].

To assess the double labeling study, 16 half-biopsies were processed (Table 2). Two successive bulk stainings were performed at room temperature on the same sample, combining basic fuchsin, fluorescein, xylenol orange and calcein green (Table 2). We considered basic fuchsin as the staining reference. For this, bone samples were bulk-stained in 1% basic fuchsin in 70, 80, 90, and 100% ethanol under vacuum at 15–20 mmHg for 4 h each. After staining, core samples were fixed in 70% ethanol, rinsed in 100% ethanol and embedded in methyl methacrylate [6, 34, 35].

The best staining results obtained on ewes were used on human trabecular bone.

Preparation of human trabecular bone

Transiliac bone biopsies

Fifty postmenopausal osteoporotic women who had been on bisphosphonates were recruited from the outpatient clinic. Transiliac bone biopsies were performed after all patients were double-labeled with demethylchlortetracycline. These samples were used to verify and validate the effect of fluorochromes on double tetracycline labels [36].

Vertebral bone cores

Six human vertebrae (3L2 and 3L4) obtained from recently deceased donors were bulk-stained for 11 days at room temperature in 5 mM xylenol orange based in 70% ethanol [37, 38]. After staining, a 8.25-mm diameter cylindrical trabecular specimen was removed using a diamond-tipped coring tool. To detect the induced microdamage, trabecular cores were bulk-stained for 2 days in 0.5 mM green calcein based in 100% ethanol and thereafter embedded in methyl methacrylate. These samples were used to verify the double labeling of fluorochromes for the detection of microdamage.

Preparation for histomorphometry

All undecalcified bones were cut along the long axis at very low speed using a heavy-duty microtome (Polycut E, Leica Microsystems, Wetzlar, Germany) equipped with tungsten carbide knives to obtain at least three non-contiguous, parallel, $100 \pm 5 \mu\text{m}$ sections for detection of microcracks. Several consecutive 8- μm sections were cut to measure bone trabecular volume and area, and also to be used afterwards to test the classical histological staining procedures. Sections were then mounted between microscope slides and cover glasses with a mounting medium (Neo-Entellan®, Merck, Germany).

Microdamage detection and analysis

Microdamage was detected by optical microscopy (Carl Zeiss, Germany) under fluorescent light at $\times 200$

¹ B660-30 JT Baker, Phillipsburg, NJ, USA.

² F 6377 Sigma Chemical Co., St Louis, MO, USA.

³ M1255 Sigma.

⁴ A3882 Sigma.

⁵ C 0875 Sigma.

⁶ 398187 Sigma.

Table 1 Staining tests performed ($n = 34$) with chelating agents including concentration, bulk staining time and final result for the detection of both microdamage and labels

Agent	Dilution	Concentration	Time	Results			Final results
				Crack stained	Crack measurable	Visibility of previous label	
Basic fuchsin	Ethanol	1%	24 h	++	++	+	+
Fluorescein	Ringer’s solution	12%	24 h	+++	+	----	–
		12%	12 h	+	----	----	----
			60 min	+	----	----	----
			30 min	+	----	----	----
		6%	24 h	+	----	----	----
	Ethanol 100%	3%	24 h	+	----	----	----
		12%	24 h	+++	+	----	----
		12%	12 h	+	–	----	----
			60 min	+	–	----	----
			30 min	+	–	----	----
		6%	24 h	+	–	----	----
		3%	24 h	+	–	----	----
Calcein blue	Water	10 mM	12 h	–	–	+	+
		0.1 mM	2 h	++	++	–	–
	Ethanol 100%	10 mM	12 h	–	–	+	+
		0.1 mM	2 h	++	++	–	–
Alizarin complexone	Water	0.5 mM	2 h	+++	+++	+	+
	Ethanol 100%	0.5 mM	2 h	+++	+++	++	++
Calcein green	Tris HCl 0.5 M	0.5 mM	12 h	–	–	–	–
			2 h	+++	+++	+	++
	Water	0.5 mM	12 h	–	–	–	–
			2 h	+++	+++	+	++
	Ethanol 100%	0.5 mM	12 h	–	–	–	–
			2 h	+++	+++	+++	+++
Xylenol orange	Tris HCl 0.5 M	1%	2 h	–	–	–	–
			12 h	–	–	–	–
	Water	0.5 mM	2 h	+++	+++	+	++
			12 h	–	–	+	–
			2 h	–	–	+	–
			12 h	–	–	–	–
	Ethanol 100%	5 mM	2 h	+++	+++	+++	+++
			12 h	–	–	+++	–
			2 h	–	–	+++	–

+, weak results; ++, acceptable results; +++, excellent results; -, no result; ---, excessive results

magnification and using morphometry software (Bone Morpho; Explora Nova, La Rochelle, France). Microdamage was observed using several specific filters at excitation/emission wavelengths of 440/610 nm (Filter set #20⁷ from Zeiss), 495/520 nm (Filter set #09⁸ from Zeiss) and

390/590 nm (Filter set #18⁹ from Zeiss) (Table 3). To avoid artifactual cutting damage, the region of measurement was located < 0.8 mm from any cutting edge. Microdamage was categorized and quantified as linear microcracks or diffuse damage. Outcome assessments included the number of linear cracks (Cr.N, #), the number of diffuse damage areas (Dx.N, #), the linear crack density,

⁷ Filter set #20: Excitation BP 546/12, Beam Splitter FT 560, Emission BP 575–640.

⁸ Filter set #09: Excitation BP 450–490, Beam Splitter FT 510, Emission LP 515.

⁹ Filter set #18: Excitation BP 390–420, Beam Splitter FT 425, Emission LP 450.

Fig. 2 All chelating agents preserved the quality of classical histomorphometric staining: **A** shows the classical histomorphometry stain: *a* Goldner's trichrome, *b* Solochrome cyanin R, *c* May-

Grünwald-Giemsa, *d* Toluidine blue, and **B** shows the corresponding stains after bulk staining by calcein green

Table 2 Two successive bulk stainings were performed on each sample ($n = 16$)

<i>n</i>	Fluorochrome no. 1	Fluorochrome no. 2	Results
1	Fluorescein	Basic fuchsin	---
2	Basic fuchsin	Xylenol orange (water)	---
3	Calcein green (ethanol)	Xylenol orange (water)	---
4	Calcein green (ethanol)	Basic fuchsin	+++
5	Xylenol orange (ethanol)	Basic fuchsin	+
6	Xylenol orange (water)	Calcein green (water)	+++
7	Xylenol orange (water)	Calcein green (ethanol)	+++
8	Xylenol orange (ethanol)	Calcein green (water)	+++
9	<i>Xylenol orange (ethanol)</i>	<i>Calcein green (ethanol)</i>	+++

Only 9 of 16 biopsies presented interpretable microcracks. Final results on the ability to see microcracks are presented. Assays retained are shown in *italics*: xylenol orange followed by calcein green (ethanol)

+++ , excellent results; + , weak results; --- , excessive results

Table 3 Dilution, concentration, wavelength and filter used for optimum results in microdamage detection

Agent	Dilution	Concentration	Excitation wavelength	Emission wavelength	Filter
Basic fuchsin	Ethanol 100%	1%	608	631	Green
Fluorescein	Ringer's solution	12%	460	515	Green
Alizarin complexone	Ethanol 100% or water	0.5 mM	580	625	Red
Calcein blue	Ethanol 100% or water	0.1 mM	375	435	Blue
Calcein green	Ethanol 100% or water	0.5 mM	495	520	Green
Xylenol orange	Ethanol 100% or water	5 mM	440, 570	610	Orange

defined as the number of linear microcracks per bone area (Cr.Dn, $\#/\text{mm}^2$), and the diffuse damage density, defined as the number of diffuse damage regions per bone area (Dx.Dn, $\#/\text{mm}^2$). Length and area of damage were expressed as mean linear crack length (Cr.Le, μm) and diffuse damage area (Dx.Ar, μm^2), whereas length and area density were expressed as linear length density (Cr.Le.Dn,

$\mu\text{m}/\text{mm}^2$) and diffuse damage area density defined as diffuse damage area per bone area (Dx.Ar.Dn, %). The reproducibility of microdamage identification was assessed by two observers independently analyzing five sections from five different donors. They identified 35 and 32 microcracks, respectively, 31 of which were concordant, providing a Kappa score of 0.77. A Kappa score is always

≤ 1 . A value of 1 implies perfect agreement and values < 1 imply less than perfect agreement. In our study, a result between 0.60 and 0.80 indicated a good agreement. The reproducibility of linear microcrack length and diffuse damage area measurements were assessed by two observers on 25 microcracks and/or diffuse damage regions. The intraclass correlation coefficients for both length and area measurements were 0.99.

Results

Ewe bone

Two of the six fluorochromes used to evaluate the best microcrack labeling were not satisfactory (Table 1). With fluorescein, microcracks were visible but tetracycline labeling (double labeling) faded and the bone area was partly covered with fluorochrome. With calcein blue staining, at low concentrations, double labeling (tetracycline) was visible but not the microdamage whereas at high concentrations, cracks were visible but double labeling was faint (histomorphometry measurement unavailable). Optimal results in dilution, concentration, wavelength and filter for microdamage detection are presented in Table 3. The classical histomorphometric stains (Goldner's trichrome, Solochrome cyanin R, May–Grünwald–Giemsa and Toluidine blue) were preserved after bulk staining by all the chelating agents. Four sections stained by classical stains after calcein green bulk staining are presented in Fig. 2. In Goldner's staining, osteoid still appears red-orange and mineralized bone green appears as before bulk staining (Fig. 2a). Solochrome cyanin R staining, which is a reliable stain giving an unequivocal identification of osteoid, calcified matrix and bone cells, is still valid with the calcified bone appearing in a uniform dark blue color and osteoid in red (Fig. 2b), and osteons are still visible in polarized light. May–Grünwald–Giemsa staining still distinguished bone marrow cells after bulk staining in calcein green (Fig. 2c). Toluidine blue still stained cells, nuclei and osteoid but the calcified bone matrix remained almost unstained (Fig. 2d). Two successive bulk stainings were performed on the same sample combining basic fuchsin, fluorescein, xylenol orange and calcein green (Table 2). Several combinations of fluorochromes allowed the detection of both the microdamage and the tetracycline. One of the best combinations was xylenol orange followed by calcein green (in aqueous or alcoholic solutions); however, aqueous solutions tended to decrease the visibility of previous labels (Table 1).

We also compared Cr.Le measurements after bulk staining by the reference, basic fuchsin, followed by calcein green (Table 4a) or xylenol orange (data not shown).

Assessment of the analyzed microdamage was similar in sections stained with both basic fuchsin and calcein green or xylenol orange. Cracks stained by basic fuchsin were longer than those stained by calcein green. For example, among seven microcracks from the same sample, we found a mean difference at $-2.01 \mu\text{m}$ (ranging from -0.68 to $-6.66 \mu\text{m}$), and a relative error in the microcrack length between -0.37 and -4.34% with a mean at -1.98% , between the measurements (Table 4a). A highly significant correlation was also observed between the two measurements ($r = 0.999$, $p < 0.001$). We compared the length of the microcracks stained by calcein green with those stained by xylenol orange in the same samples (Table 4b). On the same sample, 8 microcracks stained by calcein green appeared to be longer than those stained by xylenol orange. These data showed a difference between -3.12 and $12.42 \mu\text{m}$ with a mean of $+1.55 \mu\text{m}$, and a relative error between -11.02 and 12.90% with a mean of $+0.95\%$ (Table 4b). There was no significant difference between the lengths measured with the two fluorochromes and a highly significant correlation was observed between the two measurements ($r = 0.967$, $p < 0.001$).

Our result on ewes showed that calcein green at 0.5 mM concentration diluted in 100% ethanol as well as xylenol orange at 5 mM were the most adequate fluorochromes to detect both microdamage and to preserve the double tetracycline labeling. Examples are shown in Fig. 3. These stains were then checked on human trabecular bone.

Human bone

In iliac trabecular bone, results were consistent with those on ewes showing that the same cracks could be stained by several dyes; all microcracks stained with basic fuchsin were also observed with calcein green. Only one cross-hatched microcrack and one area of diffuse microcracks were observed. The mean cancellous bone microcrack length was $120 \mu\text{m}$.

In vertebral trabecular bone, both fluorochromes (xylenol and calcein) were distinguishable using two wavelength filters. Because all vertebrae were bulk-stained with xylenol orange and with calcein before and after cylindrical specimen preparation, respectively, the microdamage stained with xylenol orange means they were present in vivo, and those stained with calcein green were produced from drilling and sectioning. In vivo, Cr.Dn was significantly greater than artifactual damage (1.49 ± 1.25 vs. $0.41 \pm 0.35 \text{ \#}/\text{mm}^2$; $p = 0.003$).

Discussion

The purpose of this study was to adapt several staining methods developed by Norman, Schaffler, Burr, Lee, and

Table 4 Examples of results on two successive bulk stainings (same sections, same crack, with two different filters): quantification of microcracks length stained in calcein green (excitation/emission wavelength of 495/520 nm) compared to microcrack length stained with: (a) 1st sample: basic fuchsin (transmitted light), and (b) 2nd sample: xylenol orange (excitation/emission wavelength of 440/610 nm)

(a)	Crack length Calcein green (μm)	Crack length Basic fuchsin (μm)	Difference (μm)	Relative error (%)
<i>n</i> = 7				
1	55.62	56.30	−0.68	−1.22
2	74.06	75.63	−1.57	−2.12
3	27.91	29.12	−1.21	−4.34
4	209.93	216.59	−6.66	−3.17
5	97.47	99.53	−2.06	−2.11
6	207.25	208.01	−0.76	−0.37
7	208.90	210.05	−1.15	−0.55
Mean	125.9	127.9	−2.01	−1.98
SD	80.2	81.1	2.1	1.43
(b)	Crack length Calcein green (μm)	Crack length Xylenol (μm)	Difference (μm)	Relative error (%)
<i>n</i> = 8				
1	58.27	54.87	3.4	5.83
2	85.49	84.23	1.26	1.47
3	66.86	74.23	−7.37	−11.02
4	42.46	45.58	−3.12	−7.35
5	128.34	121.62	6.72	5.24
6	72.13	79.22	−7.09	−9.83
7	59.77	53.59	6.18	10.34
8	96.25	83.83	12.42	12.90
Mean	76.20	74.65	1.55	0.95
SD	27.63	25.78	7.03	8.55

(a) A mean difference of ~2 μm is found in the linear microcracks length between the two stains, but a significant correlation is observed ($r = 0.999$, $p < 0.001$).

(b) A mean difference of ~1.6 μm is found in the linear microcracks length between the two stains but a significant correlation is observed ($r = 0.967$, $p < 0.001$).

O'Brien et al. [2, 3, 6, 16, 17, 19, 29, 31] to our specific aim, i.e., the detection of both microdamage and fluorochrome labels (tetracycline) in human bones. Most of the previous tests using several dyes were performed on cortical bone. The novelty of our study was the use of several fluorochromes to detect microdamage in trabecular bone without deleting fluorochrome labels, with a large study in ewes and validation in human bones used in clinical practice (iliac bone).

Fluorochromes are calcium binding substances that are preferentially taken up at the site of active mineralization of bone, also known as the calcification front. Sun et al. [39] found no differences between either of the non-tetracycline labels (xylenol orange and calcein green) for any of the histomorphometric measurements. In our study, we found that, compared to the basic fuchsin staining, microcracks measured by calcein green were slightly smaller in length (mean 2 μm), but both measurements were highly correlated. This could be due to the fact that fuchsin-stained microcracks were measured under transmitted light. Lee et al. compared fuchsin-stained microcracks in human rib bones using transmitted light and epifluorescence microscopy. They found no differences between the two methods in crack number, density or length indicating comparable accuracy [17], suggesting that our epifluorescence

microscopy measurements could have provided similar results. The mean difference between the microcrack length observed with calcein green bulk staining followed by xylenol orange was 1.55 μm in our study, without predominance of one stain on the length of the microcracks. This difference is too small to be clinically relevant. Comparatively, in compact bone, Lee et al. [31] found that the length [mean (SD)] of microcracks in ten rib bones was 70.30 (46.87) μm in calcein green and 62.58 (46.45) μm in xylenol orange without a significant difference. O'Brien et al. provided one possible explanation about the length difference between the two fluorochromes. They measured the affinity of chelating agents for Ca^{2+} ions with chromatography and found that the mean height of calcium peak was higher in calcein green (0.945) than xylenol orange (0.88). The smallest number (xylenol, 0.88) indicated a greater affinity for calcium than calcein green [40]. Alternatively, as these differences were small, they may stem from measurement variability and would therefore not be significant.

In our study, we also found little diffuse damage in both ewe bones and human iliac bones. This could be due to the fact that, in both cases, the iliac bone is not a weight-bearing bone. Moreover, >50% of human bone biopsies had no visible trabecular bone microcracks. In

Fig. 3 Examples of successive staining on ewes using several filters: **a** and **b** microcrack stained by basic fuchsin stays visible after calcein green staining using a 546/640 filter (Filter set #20), **c** and **d** double tetracycline label (*down*) and a microcrack (*upper*) are visible using a 390/450 filter (Filter set #18) but only microdamage (*upper*) remains visible after fluorescein staining using a 450/515 filter (Filter set #09), **e** and **f** microcrack stained in alizarin using a 546/640 filter (Filter set #20) is no longer visible (*right*) with a 450/515 filter (Filter set #09)

the only study comparable to ours, Stepan et al. [41] measured transiliac crest biopsies from 38 women who had been treated with alendronate for an average of 5 years, and compared them to a control sample of 28 women who were treatment naive. They found a difference in the mean crack length (μm) of approximately $47 \mu\text{m}$ between the treatment-naïve and alendronate-treated women. The crack length they observed was shorter than in our study. This may be explained by false-positive findings. Indeed, some smaller structures—like osteocyte canaliculae—can be reminiscent of the appearance of cracks and can be mistaken for cracks. Microdamage was also rare in cortical bone, and only three samples had at least one observable microcrack [36]. This may also stem from the type of bone we studied. In animal models, it has been shown that most microcracks are observed in long weight-bearing bones, e.g., the femurs. In addition, the clinical significance of microcracks may differ between cortical and trabecular bone and whether the microdamage occurs under physiological or experimental conditions. Indeed, microdamage

induced by fatigue loading impairs bone mechanical properties, whereas pre-existing physiological microdamage observed in vertebrae does predict mechanical properties [37].

It is important to remember that alcohol influences bone mechanical properties, hence crack propagation. Therefore, we used a combination of calcein green at 0.5 mM concentration and xylenol orange at 5 mM, which preserved bone as much as possible, and also allowed the use of other techniques to explore bone quality (data not shown: microradiography [42], Fourier transform infrared microscopy [43], microhardness [44]).

In conclusion, this methodological approach allowed the detection and the identification of both microdamage (linear, diffuse and cross-hatched)—pre-existing *in vivo* in bone, or induced by the technique or mechanical loading—and tetracycline labels as demethylchlortetracycline. Calcein green at 0.5 mM concentration and xylenol orange at 5 mM seem to be the most appropriate dyes to be able to study both microcracks and histomorphometry because they preserve the tetracycline labels.

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Conflict of interest All the authors state that they have no conflicts of interest.

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