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ORIGINAL ARTICLE

Effect of a formalin-based fixation method on bone mineral content in human *ex-vivo* specimens

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ABSTRACT

Objective: Histopathology of formalin-fixated human *ex-vivo* specimens may be used as reference standard for evaluation of diagnostic index tests like CBCT or MRI. The aim was to estimate changes in bone mineral content (BMC) over time in human *ex-vivo* bone specimens fixated in a formalin-based solution for 24 h followed by storage in an alcohol-based medium for six months, assessed by dual-energy X-ray absorptiometry (DXA).

Methodology: Bone specimens ($n = 19$) from human *ex-vivo* mandibles donated for science were included. BMC was measured by DXA before fixation (D_0), after 24 h of immersion fixation in a formalin-based solution (D_1), and hereafter every 30 days (M_1-M_6) during storage in a 30% ethanol-based storage medium for 6 months. Changes in BMC from D_0 to D_1 and from D_0 to M_6 were calculated and mean change in BMC estimated.

Results: Mean change in BMC from D_0 to D_1 was −0.73% (95% CI −1.75%; 0.29%), and from D_0 to M_6 −1.19% (95% CI −2.14%; −0.23%).

Conclusions: No changes in BMC of *ex-vivo* human bone specimens were found after 24 h formalin-based immersion fixation. After six months storage in an ethanol-based medium, BMC mean loss of 1% was detected. In this range, changes in BMC are not clinically relevant.

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Reference standard; bone mineral content; DXA; formalin fixation; *ex-vivo* human specimen

Introduction

During the last two decades, cone beam computed tomography (CBCT) has often been used for diagnosis of apical periodontitis (AP) and other diseases related to pathologic bone mineral loss, as well as for treatment planning in e.g. endodontic and surgical practice [1–3]. Recently also magnetic resonance imaging (MRI) has been proposed and evaluated for diagnosis of AP [4–6].

Since Fryback & Thornbury introduced a model for evaluating the diagnostic efficacy of an imaging method at different efficacy levels, their hierarchical model has been used to describe and assess the diagnostic efficacy at six levels (F&T-level 1-6) [7]. The diagnostic efficacy of both CBCT and MRI used for diagnosis of AP has mainly been assessed at the lower efficacy levels (F&T-levels 1 and 2), and most studies applying CBCT have used artificially induced periapical lesions as reference standard [8–10]. However, artificially induced 'lesions' do not reflect true periapical disease. To estimate the diagnostic accuracy of an index method, e.g. radiography, pertaining to detect e.g. AP, a *Gold Standard* or *reference standard* for periapical disease is needed [11]. Histopathological examination of the periapical area, including both root and surrounding bone, is considered the reference standard for the presence of AP. However, due to ethical considerations, human *in-vivo* biopsies of the periapical area cannot be obtained in most clinical situations. Therefore human dentate *ex-vivo* jaw bone specimens are the best possible option for developing a reference standard for AP in studies of accuracy of an index method [12].

Previous studies of AP have used non-fixated human *ex-vivo* specimens as histopathological reference for a radiographic diagnosis [13–16]. Non-fixated specimens have an inborn time limitation due to *post mortem* decomposition of the body. To control *post mortem* decomposition, formalin-fixation of the tissues can be performed [17]. Fixated *ex-vivo* human specimens were utilised in a previous study with a histopathological reference of AP [18], and recently a study on the diagnostic accuracy of CBCT for diagnosis of AP was performed with human *ex-vivo* specimens intravenously perfusion fixated with a formalin-based solution as reference standard [19].

Formalin-based fixation solutions have a pH-value of 3.0–4.6 and a loss of minerals from e.g. bone tissues can therefore be expected when applied for fixation and storage. If bone mineral is lost, there is a risk for overestimating pathological bone loss. Hence, it is of importance to investigate whether this expected fixation-induced loss of bone mineral may impair the radiographic outcome when histopathology of such fixated bone specimens are used as reference standard for e.g. AP. In a study assessing the mineral loss after formalin-fixation measured by dual-energy X-ray

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absorptiometry (DXA) in full bodies, femoral bones, and lumbar vertebrae, only negligible differences in the range of few percent were reported [20]. To minimise the bone mineral loss, it has been hypothesised that the pH-value of the formalin-based fixation solution may be buffered before use. One study has assessed the effect of buffering on bone mineral loss from rabbit tibiae fixated and stored in different buffered 10% formalin solutions. This assessment was based on changes in radiographic optical density due to loss of bone mineral. A continuous loss of bone mineral over time with the greatest loss within the first 24 h was reported. Hence, the authors concluded, that fixation and storing of bone specimens using a formalin-based solution may negatively influence the radiographic image quality [21]. These results have led other authors to question the usability of formalin-fixated specimens for studies on the diagnostic accuracy of radiographic diagnosis of AP [14,15].

Bone mass measurements can be either bone mineral content (BMC) or bone mineral density (BMD). BMC displays the total amount of bone mineral in a specific area measured in gram (g), whereas BMD displays the amount of bone mineral in bone tissues per areal (g/cm^2) or volume (g/cm^3). The reference standard for assessment of BMC and areal BMD (aBMD) *in-vivo* is DXA, but DXA can also be applied for assessment of BMC and aBMD *ex-vivo* or *in-vitro* [22].

In an *in-vitro* study on the threshold value of radiographically detectable loss of bone mineral in periapical radiographs (PR), Nackerts et al. [23] concluded that the minimum proportion of bone mineral (density) loss detectable was 6.6% using a digital software-based evaluation method. However, the naked-eye can only detect bone mineral loss in PR if the loss is substantial, approximately 30% or higher [23,24].

The aim of the present study was to estimate possible changes in BMC over time in human *ex-vivo* bone specimens fixated in a formalin-based solution for 24 h and hereafter stored in an alcohol-based storing medium for six months, assessed by DXA.

Material and methods

Ethical considerations

A general request for ethical approval was sent to the Regional Committee of Ethics (Region Midt, Denmark). The committee decided that projects performed on anonymous human *ex-vivo* specimens did not need an ethical approval, as all the involved human specimens were non-identifiable to the investigators (Regional Committee of Ethics, Region Midt, Denmark, request no. 279/2017).

Collection and preparation of bone specimens

The study material consisted of human jawbone specimens from persons who had donated their deceased bodies to science at Department of Biomedicine, Health, Aarhus University, Denmark (May 2017). Within 24 h from the time of death, edentate mandibles from two donors were collected. After removal of soft tissues, the mandibles were sectioned perpendicular to the buccal bone into 1 cm blocks to make them fit into the cup holder of the high-resolution micro-CT scanner. Bone sectioning was performed using a band saw (EXACKT Pathology Saw, EXAKT Technologies Inc., Oklahoma City, OK). To include specimens with different proportions of cortical and cancellous bone, blocks from different areas of the mandible were selected. A total of 19 jawbone blocks from two donors were included (donor 1; 14 bone blocks, donor 2; 5 bone blocks). Figure 1 displays high resolution images of an included bone specimen. No information of the gender, age, cause of death, etc. was available to the investigators.

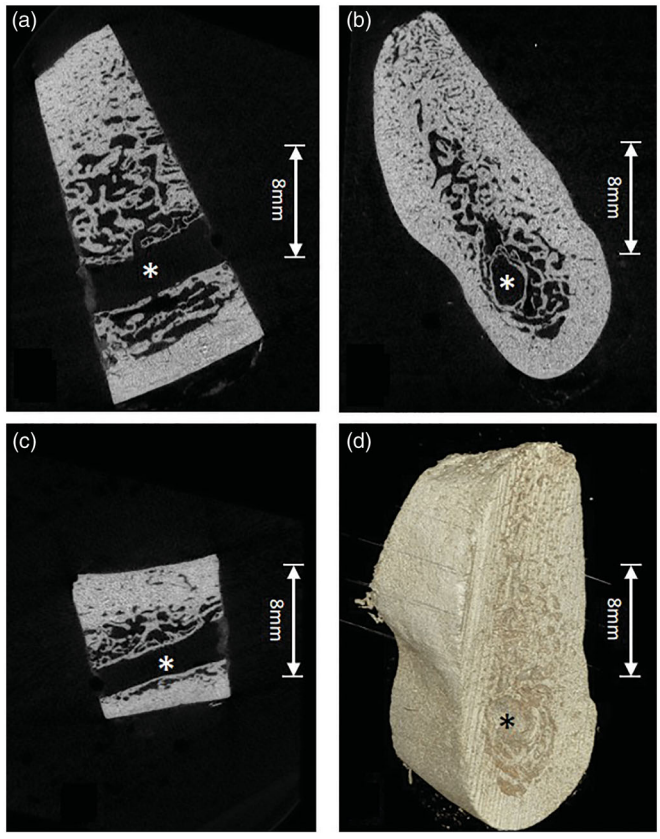

Figure 1. Example of bone specimen, left side mandibular molar region, micro-CT sections and 3 D-reconstruction. (a) Sagittal, (b) Coronal, (c) Axial, (d) 3D-reconstruction. *Mandibular canal.

Timeline, scanning, fixation, and storing

Prior to the first DXA imaging, the specimens were stored individually and dry at −18 °C. At day_0 (D_0), all bone specimens were scanned with DXA to measure BMC prior to fixation, which was performed immediately after imaging. During fixation and storage, each specimen was stored in a 100 mL plastic container. Standard fixation solution used for fixating *ex-vivo* human specimens at Department of Biomedicine, Health, Aarhus University, Denmark was used. Immersion fixation was performed using a formalin-based fixation solution (formaldehyde 24% (0.8 L), ethanol 96% (5.0 L), glycerine (2.5 L) and demineralised water (2.0 L)). After 24 h of immersion fixation, a DXA scan to measure BMC at day_1

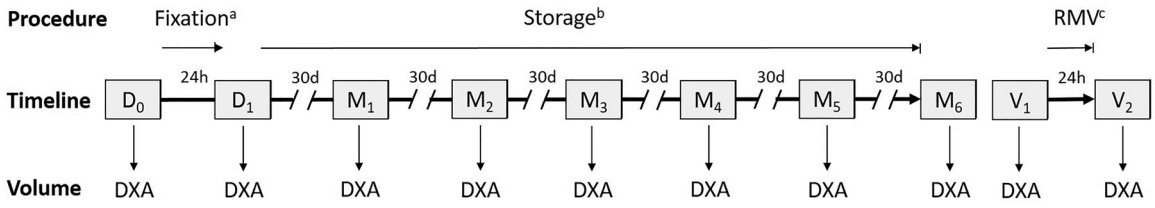

Figure 2. Timeline of procedures and DXA volume acquisitions. [a]Immersion fixation in formalin-based medium, [b]Storage in ethanol-based medium (180 days), [c]Measurement of random measurement variation (RMV), D_0: day 0; D_1: day 1; M_1-M_6: month 1–6; V_1: day-to-day variation 1; V_2: day-to-day variation 2; DXA: dual-energy X-ray absorptiometry.

(D_1) was performed. Then the specimens were stored in a 30% ethanol-based storage medium (ethanol 96% (3.12 L) demineralised water (6.8 L), 5 g potassium sortate) for 6 months. DXA scans and measurements of BMC were repeated every 30 days, month$_{1\text{-}6}$ (M_1-M_6). All DXA-based measurement of BMC was performed by a pDEXA (Sabre XL, Norland Stratec, Pforzheim, Germany) using a pixel size of $0.2 \times 0.2\text{mm}^2$ and scan speed of 3 mm/s. Quality assurance was performed using two solid-state phantoms as described by the manufacturer. Figure 2 shows a timeline of procedures and acquisitions.

Data treatment and analysis

The null hypothesis (H_0) tested was that no changes in BMC will take place over a time period of six months in human *ex-vivo* jawbone specimens after fixation in a formalin-based solution for 24 h and followed by storage in an alcohol-based medium for six months.

With an assumed standard deviation (SD) of BMC assessed by DXA at 6% [20,25,26], a sample size of 18 specimens was calculated to be sufficient to detect a change in BMC of at least 5% with a level of significance of 0.05 ($\alpha = 0.05$) and power of 90%. The final sample included 19 bone specimens.

All data were registered in an Excel spread sheet (Microsoft Inc., Redmond, WA). Data description and data analysis were performed with the individual bone specimen (or donor) as the unit of analysis. Changes in BMC over time were calculated, and mean values estimated with 95% confidence intervals (95% CI). To assess the precision of the reference standard test (DXA), two day-to-day DXA scans of BMC, variation 1 and 2 (V_1 and V_2), were performed with an interval of 24 h after time M_6. Based on these day-to-day measurements, the coefficient of variation (CV) (CV = 100x(standard deviation)/(mean value of set)) was calculated and the random measurement variation estimated. Stata 14 (Stata: Release 14. Statistical Software. College Station, TX: Stata Corporation 2015) [27] was used for all statistical analyses.

Results

No statistically significant differences between the specimens from the two donors were found, as the estimated changes in BMC with 95% CI of donor 1 were within the 95% CI of donor 2. The changes in BMC, D_0 to D_1 and D_0 to M_6, for all specimens are shown in Table 1. Figure 3 displays graphs of BMC over time in the individual bone specimens measured by DXA. As can be seen from the graphs, changes in BMC of the individual specimens are modest and all graphs stable.

Table 1. Bone mineral content (BMC) measured by DXA.

	DXA				
	BMC (g)			Change (%)	
	D_0	D_1	M_6	$D_0 - D_1$	$D_0 - M_6$
Specimen					
1	2.697	2.792	2.721	3.52	0.89
2	2.870	2.936	2.959	2.30	3.10
3	1.198	2.031	1.996	2.52	0.76
4	1.977	1.962	1.882	−0.76	−4,81
5	2.049	2.039	2.039	−0.49	−0.49
6	2.191	2.190	2.185	−0.05	−0.27
7	1.966	1.958	1.940	−0.41	−1.32
8	2.582	2.550	2.526	−1.24	−2.17
9	2.376	2.396	2.360	0.84	−0.67
10	4.111	4.051	4.000	−1.46	−2.70
11	3.533	3.472	3.497	−1.73	−1.02
12	1.934	1.965	1.937	1.60	0.16
13	2.232	2.215	2.209	−0.76	−1.03
14	1.959	1.872	1.942	−4.44	−0,87
15	1.415	1.353	1.319	−4.38	−6.78
16	0.822	0.811	0.821	−1.39	−0.15
17	3.744	3.731	3.723	−0.35	−0.56
18	2.202	2.117	2.158	−3.86	−2.00
19	0.915	0.884	0.891	−3.38	−2.60
Mean				−0.73	−1.19
95% confidence interval				[−1.75; 0.29]	[−2.14; −0.23]

BMC: bone mineral content; vBMD: volumetric bone mineral density; D0: day 0; D1: day 1; M6: month 6; DXA: dual-energy X-ray absorptiometry.

The mean change in BMC from D_0 to D_1 was −0.73% [−1.75%; 0.29%] (range −4.44% to 3.52%), so the mean change was not significantly different from 0%. The mean change in BMC from D_0 to M_6 was −1.19% [−2.14%; −0.23%] (range −6.78% to 3.67%), so the mean change differed significantly from 0%. The precision of the individual DXA measurements, expressed as CV, was calculated to be 2.1%. The random measurement variation was estimated to −0.61% [−1.83; 0.61] being not significantly different from 0%. Table 2 displays the day-to-day variation measurements for all specimens.

Discussion

In the present study, changes in BMC after formalin fixation processing of *ex-vivo* human bone specimens were assessed. No significant change in BMC was found after 24 h of immersion formalin fixation and this part of the H_0-hypothesis could not be rejected. After six months of storage in an ethanol-based storage medium an estimated decrease in BMC of approximately 1% was found, which means that this part of the H_0-hypothesis had to be rejected.

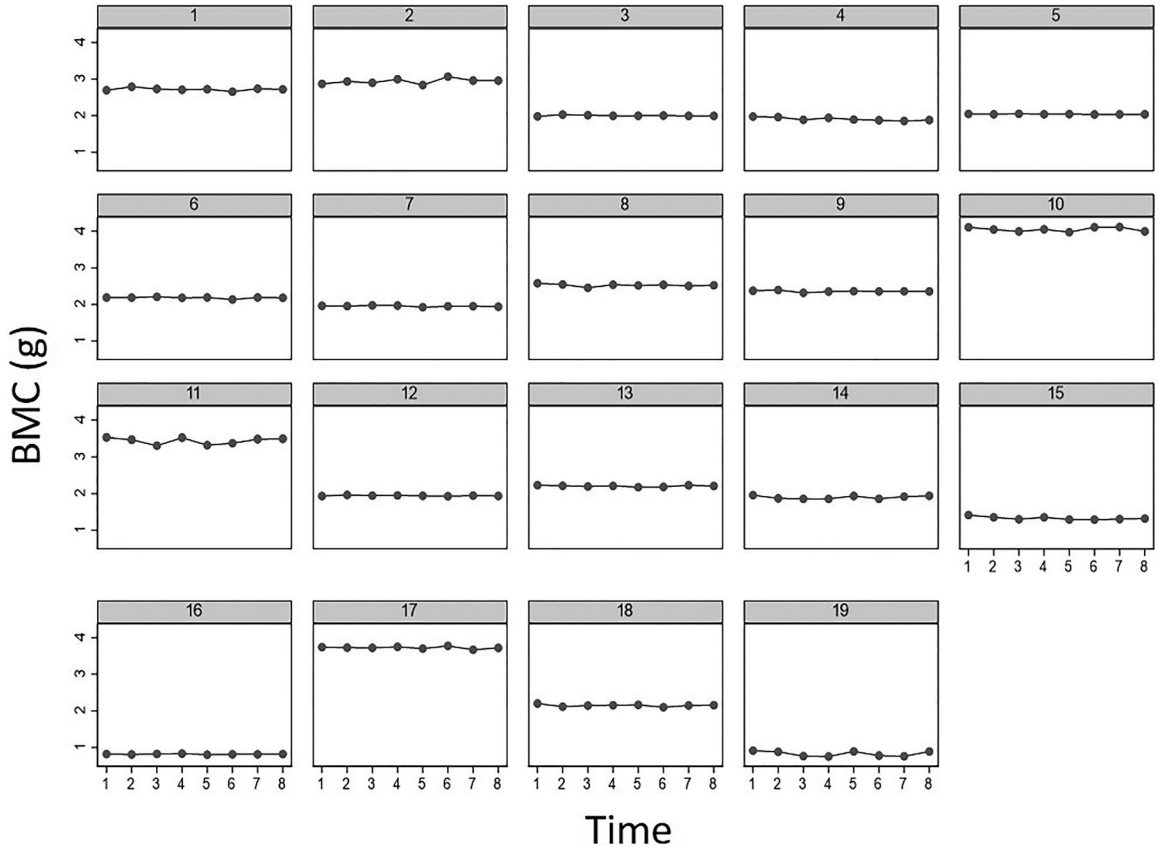

Figure 3. BMC (g) over time for individual specimens (no. 1–19). X-axis: 1 = D_0, 2 = D_1, 3 = M_1, 4 = M_2, 5 = M_3, 6 = M_4, 7 = M_5, 8 = M_6, BMC: bone mineral content; D_0: day 0; D_1: day 1; M_1-M_6: month 1–6.

Table 2. Day-to-day variation of bone mineral content (BMC) measured by DXA.

	DXA		
	BMC (g)		Change (%)
	V_1	V_2	RMV ($V_1 - V_2$)
Sample			
1	2.731	2.700	−1.14
2	2.953	2.956	0.10
3	2.005	2.001	−0.20
4	1.887	1.954	3.55
5	2.020	2.019	−0.05
6	2.166	2.192	1.20
7	1.941	1.951	0.52
8	2.522	2.523	0.04
9	2.342	2.254	−3.76
10	4.064	3.841	−5.49
11	3.487	3.443	−1.26
12	1.943	1.933	−0.51
13	2.120	2.118	−0.09
14	1.949	1.866	−4.26
15	1.278	1.271	−0.55
16	0.787	0.829	5.40
17	3.656	3.637	−0.52
18	2.154	2.037	−5.43
19	0.770	0.776	0.82
Mean			−0.61
95% confidence interval			[−1.83 ; 0.61]

BMC: bone mineral content; DXA: dual-energy X-ray absorptiometry; RMV: random measurement variation; V1: day-to-day variation measurement 1; V2: day-to-day variation measurement 2.

When the diagnostic accuracy of any given imaging method is assessed, as part of the evaluation of the overall diagnostic efficacy, a reliable reference standard for the true state of disease is needed [7]. To validate the radiographic diagnosis of AP, histopathology is considered the reference standard. When performing surgical endodontic retreatment on teeth diagnosed with AP, the periapical tissues are removed during surgery, and previous studies have evaluated the diagnostic validity/accuracy of the preoperative diagnosis using *in-vivo* biopsies as a histopathological reference [12,28–34]. Consequently, the majority of clinical studies lack a histopathological reference for negative diagnoses, and only the sensitivity can be assessed. Furthermore, performing bone biopsies is considered unethical if surgical endodontic retreatment is not part of the planned standard treatment procedure. *Ex-vivo* specimens with both naturally developed pathological lesions and healthy periapical areas are considered the second-best option for establishing a reference standard [11], because it reveals the natural disease phases of AP, contrary to *in-vitro* references based on artificially induced lesions [8].

The primary advantage of fixated specimens is that they can be stored and handled in several imaging procedures, e.g. in different scanners, without degeneration or structural changes in the tissues prior to histopathological evaluation. Previous *ex-vivo* studies have evaluated radiographic diagnosis of AP against histopathology using specimens not fixated before acquisitions of images [13–16]. One study using formalin-fixated specimens did not describe the fixation method [18], and one recent study was based on specimens fixated by perfusion of a formalin-based medium before radiographic imaging [19]. Hence, it is of importance to evaluate

whether the fixation process with formalin impairs the reliability of radiographic diagnostics of pathological bone loss due to a decrease in bone mass induced by the fixation medium.

The precision of the reference test (DXA) in the present study was very good as CV was only 2.1%. This high precision is comparable to the CVs for DXA measurements by Nackaerts et al., [23] which were all below 3%. The estimated mean change in BMC from D_0 to M_6 of −1.19% [−2.14%; −0.23%] is comparable to findings by Lochmüller et al. [20] who reported changes in BMC of −2.0% (full body), −0.6% (femur) after perfusion fixation by a formalin-based medium and 10 months storage in an ethanol-based medium. In their study performed on formalin immersion-fixated rabbit tibiae, Fonseca et al. [21] reported a loss in radiographic optical density, due to loss of bone mineral, but also at a level way lower than what is detectable by the naked eye radiographic assessment.

In the present study, BMC was chosen to assess the possible change in bone mineral after fixation in a formalin-based medium. BMC defines the total content of bone mineral in grams and is hence a measurement independent of other parameters of the specimen. aBMD (g/cm^2) would also have been a possible measurement, but is highly dependent on the positioning of the specimen in the scanner [22]. Further, aBMD is also susceptible to change in the specimen volume due to formalin-induced shrinkage, though minimal for non-decalcified bone specimens [35–37], and may be a less precise measurement for comparisons over time.

Access to *ex-vivo* human material depends on persons, who donate their future deceased body to science. The material is invaluable, and the handling procedures are both extensive and expensive. For educational and scientific purposes, the bodies need to be fixated and preferably intact, and harvesting of fresh bone blocks may limit the usability of the rest of the donated body. Bone specimens from two donors were used in the present study. Statistical comparison of the mean changes in BMC between the two donors showed no statistically significant difference in the specimens from the two donors, and hence no further analyses of the individual donors were performed.

Fixation of tissues can be performed in different ways. Perfusion fixation is performed on the full body with an intact blood vessel system by injecting a fixation solution just after death, whereas immersion fixation can be applied to smaller tissue samples by simple immersion into the fixation medium. Because of the above-mentioned limitation in donor bodies as well as the limited FOVs in the applied DXA unit, the bone specimens included in the present study were fixated by immersion. The time frame of evaluating the possible change in BMC over a six month period was selected, because a full fixation process by perfusion fixation is first completed after the six month storage in a storage medium.

A recent study applied the exact same fixation and storage mediums, but the included specimens were perfusion fixated [19]. Due to the buffer effect from different tissues in a full body, the possible change in bone mass induced by formalin is assumed to be smaller for perfusion-fixated than for immersion-fixated specimens, where dissolution of minerals into the surrounding liquid is unimpeded. Hence, it is expected that perfusion-fixated specimens would be as reliable as immersion-fixated specimens when histopathology is used as reference standard for evaluation of index tests. Based on this and the negligible fixation-induced changes in bone mass, it may be concluded that formalin-fixated human jaw specimens can be used when assessing radiographic detection of AP, or other pathological bone loss, with histopathology as the reference standard without the risk of over-diagnosis.

Conclusions

No changes in BMC of *ex-vivo* human jaw specimens were found after 24 h immersion fixation in a formalin-based solution. After further six months of storage in an ethanol-based storing medium, an estimated mean loss in BMC of approximately 1% was detected by DXA. In this range, changes in BMC are not clinically relevant when histopathology of formalin-fixated specimens are used as reference standard for evaluation of index tests. The formalin-fixated specimens can reliably be applied without the risk for overestimating pathological bone mineral loss.

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Disclosure statement

The authors report no conflicts of interest.

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