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Accuracy of MicroCT in the Quantitative Determination of the Degree and Distribution of Mineralization in Developing Bone

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Purpose: To evaluate the accuracy and applicability of a commercially available microCT system for comparative measurements of the degree and distribution of mineralization of developing bone.

Material and Methods: Homogeneous K$_2$HPO$_4$ solutions with different concentrations (range 0–800 mg/cm$^3$) were used to assess the accuracy of a microCT system equipped with a polychromatic X-ray source. Both high (45 kV) and low (70 kV) tube peak voltages were explored. The resulting attenuation was compared with calculated theoretical attenuation values to estimate the accuracy. As an example of its applicability, the method was used to assess changes in the degree of mineralization of various regions of the mandible from two pigs of different developmental age.

Results: On average, the estimated error of the measured linear attenuation was 10% or less. Accuracy was dependent on the average mineral concentration, the size of the sample, and the energy of the X-ray beam. The accuracy of the microCT system appeared sufficient to distinguish regional differences in the degree of mineralization within and between specimens of developing mandibular bone. Furthermore, the resolution of the system allowed identification of different degrees of mineralization within trabeculae.

Conclusion: Accuracy of microCT with polychromatic radiation can be considered adequate for assessment of the degree of mineralization of developing bone. Therefore, this method provides a three-dimensional means by which to simultaneously investigate the bone structure as well as the degree of mineralization during development in a non-destructive manner and with high resolution.

Key words: Bones; growth and development; bones, mineralization; CT, artifact; CT, experimental studies; micro-computed tomography

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Along with architecture, bone mineralization has proved to be a key factor in determining the quality and mechanical properties of trabecular bone (2, 6, 22). There are several techniques applied to quantify the structure and/or mineralization of bone during its development in fetuses and newborns, among them histology (13), dual energy X-ray absorptiometry (DXA (4, 19, 20, 25)), quantitative computed tomography (QCT (3)), synchrotron radiation computed tomography (16), and backscattered electron microscopy (BSE (23, 26)). The resolution of DXA and clinical QCT is insufficient to reveal the degree of mineralization at trabecular level, whereas histology and BSE require destruction of the investigated object and can only visualize the object in two dimensions. Synchrotron radiation computed tomography, with its unbiased assessment of the degree of mineralization, its high resolution, and its non-destructive measurement, is time-consuming and has limited availability. In the past few years, micro-computed tomography (microCT) has been used extensively to quantify the micro-architectural properties of trabecular bone three-dimensionally (24). Bone volume fraction, trabecular number,
thickness, separation, and anisotropy, for example, have proved to be fundamental characteristics in describing the trabecular structure of bone (e.g. adult bone (1, 9, 12) and postnatal development (14, 27)). However, thus far the possibilities for the application of microCT in the quantification of the degree of mineralization of bone have not been explored.

Unambiguous quantification of the degree of mineralization using microCT is not straightforward owing to the polychromatic character of the X-ray beam in commercially available microCT systems. The disadvantage of such a polychromatic beam (the X-ray beam contains a broad spectrum of energies) is that low energy (“soft”) radiation is more readily absorbed than high energy (“hard”) radiation, which causes the energy spectrum of the beam to change as it passes through the investigated object (5). When the beam has passed through the object, it contains a relatively large portion of high energy (“hard”) radiation, hence this effect is known as beam-hardening. The high absorption of soft radiation at the outer region of an object gives the impression of a higher mineral concentration in this portion in comparison with the internal region, where the remaining hard radiation is not so readily absorbed, causing an underestimation of the mineral concentration in this section of the object (Fig. 1A–C). Moreover, beam-hardening leads to a non-linear relationship between the linear attenuation of radiation and the material density and object dimensions. The attenuation becomes more underestimated with increasing density and object size (Fig. 1D). As this effect is well known, a microCT apparatus is commonly equipped with a beam-hardening correction algorithm, which reduces the non-linearity of the relationship by fitting it with polynomials (10).

The goal of this study was to evaluate the accuracy and applicability of a microCT system,
equipped with a beam-hardening correction algorithm, to quantify the degree of mineralization as well as its distribution in developing bone. As the beam-hardening artifact becomes stronger when more radiation is absorbed, it is likely that these measurements are much less influenced by beam-hardening than mature bone because of a relatively low degree of mineralization in developing bone. The developing mandible has been chosen to assess the applicability of the method in an environment with non-homogeneous mineral density distribution. As this structure exhibits a complex timing of ossification, it can be expected that in each stage of development a wide range of mineralization densities will be present.

Material and Methods

MicroCT
The degree of mineralization was analyzed with a high-resolution microCT system (µCT 40; Scanco Medical AG., Bassersdorf, Switzerland). This microCT system is based on an X-ray tube that produces a cone-shaped beam which is detected by a CCD detector (2048 x 64 elements; 2048 x 2048 pixels in image matrix). A detailed description of the system can be found elsewhere (24). Cross-sections were reconstructed using the Feldkamp algorithm (8).

The analysis was performed at low (45 kV) and high (70 kV) peak voltages of the X-ray tube. Furthermore, a high current and a low current setting for each peak voltage was evaluated. The X-ray beam was prefiltred with an aluminium filter (0.5 mm) to remove the softest rays. Reduction of the effect of beam-hardening was accomplished by applying a correction function to the detected X-ray radiation attenuation, which was developed by the manufacturer. For imaging purposes, each voxel was depicted with a gray value, which was related to the linear attenuation. It was assumed that this attenuation was proportional to the degree of mineralization.

Reference measurements
The mineral hydroxyapatite (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH\textsubscript{2})) is the main constituent in calcified bone that causes X-ray attenuation. The degree of mineralization is therefore assumed to be equivalent to the concentration of hydroxyapatite. Owing to the solubility problems, it was impossible to obtain a homogeneous solution of this mineral for calibration purposes. As the water-soluble dipotassium hydrogen phosphate (K\textsubscript{2}HPO\textsubscript{4}; Merck, Darmstadt, Germany) displays the same absorption characteristics as hydroxyapatite over a wide range of energies (17), it was utilized for calibrating the linear attenuation coefficients and determining the accuracy of the method.

The influence of the available beam-hardening correction algorithm was illustrated by comparing the linear attenuation for a series of K\textsubscript{2}HPO\textsubscript{4} reference solutions of different concentrations in a sample holder of 20 mm outer diameter (Plexiglass, wall thickness 1.5 mm) with and without implementation of the beam-hardening correction algorithm. These were scanned using a low energy X-ray beam (45 kV, 177 µA combination). For each concentration, the average attenuation was calculated over a circular cylinder in the center of the sample, with a diameter approximately three-quarters of the total inner diameter of the sample. Here the linear attenuation values are approximately constant.

Reference measurements were performed with cylindrical specimen holders with different outer diameters (12, 20, and 36 mm), filled with different solutions of K\textsubscript{2}HPO\textsubscript{4} (0, 10, 50, 150, 250, 375, 500, and 800 mg/cm\textsuperscript{3}). Four energy-current settings were applied, namely low energy (45 kV with 88 µA and 177 µA) and high energy (70 kV with 57 µA and 114 µA). All the reference measurements were performed with application of the polynomial beam-hardening correction algorithm. The average linear attenuation of a specific concentration of K\textsubscript{2}HPO\textsubscript{4} was determined for the central cylindrical portion with a diameter of three-quarters of the inner diameter of the sample. For each specimen holder diameter-energy combination, a relationship between K\textsubscript{2}HPO\textsubscript{4} concentration and linear attenuation was obtained. By fitting these curves with 2nd-order polynomials, calibration curves for each specimen diameter-energy combination were established.

Accuracy
In order to assess the accuracy of the recorded linear attenuation, the measured relationship between attenuation and K\textsubscript{2}HPO\textsubscript{4} concentration of the reference solutions was compared with the theoretical relationship. Calculation of the theoretical linear attenuation coefficients for a specific mixture is based on the concept of additivity, according to which the mass attenuation coefficient for a mixture [(µ(E)/ρ)\textsubscript{mixture}] with atomic constituents \textit{i} for a given energy can be determined according to:

\[
\left( \frac{\mu(E)}{\rho} \right)_{\text{mixture}} = \sum_i w_i \left( \frac{\mu(E)}{\rho} \right)_{i}
\]

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where $w_i$ is the fraction by weight of the $i^{th}$ atomic constituent. For each concentration of K$_2$HPO$_4$ in water, there are different ratios between the atoms in the mixture, which leads to different mass attenuation coefficients for the specific mixtures. The mass attenuation coefficients of the different mixtures of K$_2$HPO$_4$ with water were obtained from the National Institute of Standards and Technology (NIST) databases (http://physics.nist.gov/PhysRefData) with the effective energy representing the total radiation spectrum. These effective energies were estimated by taking the average linear attenuation value in the center of a cross-section through a K$_2$HPO$_4$ solution. The energy corresponding to this attenuation value was looked up in the NIST databases (11) and treated as the effective energy.

In order to obtain a three-dimensional visualization of the mandibular bone, it was digitally separated from the background, soft tissues, and cartilage in the resulting attenuation maps by applying global thresholds (7). Every voxel below this attenuation threshold was made transparent, whereas the remaining voxels, belonging to bone, were made opaque. For quantification of the degree of mineralization, the same global threshold was applied in the three-dimensional attenuation maps, to remove all soft tissue, while the attenuation values of all bony matter remained unaffected.

Several volumes of interest, approximately $1 \times 1 \times 0.5 \text{mm}^3$ (resolution 10 $\mu\text{m}$) in size, were defined to compare the regional degree of mineralization of both mandibular specimens. They were selected in the symphyseal region, mandibular corpus, mandibular ramus, mandibular angle, coronoid process, condylar neck, and condylar head (Figs. 4 and 5). The degree of mineralization was calculated as the average mineralization density of the bone within a volume of interest using the calibration lines obtained from the reference measurements. Accuracy of the measured degree of mineralization of the bone was calculated according to the accuracy of the reference measurements.

Results

Reference measurements

The cross-sectional images in Fig. 1A and B illustrate the influence of the beam-hardening correction function in a 500 mg/cm$^3$ K$_2$HPO$_4$ solution. The characteristic cupping profile (Fig. 1A) with the relatively high absorption of (soft) radiation at the edges and less absorption (hard radiation) in the inner region was clearly reduced when beam-hardening correction was used (Fig. 1B). The line profiles through the cross-sectional images in Fig. 1A and B also show that the obtained attenuation varied throughout the entire specimen, in contrast to its theoretical homogeneous mineral density indicated by the blue line in Fig. 1C. Therefore, in order to quantify the accuracy of the reference measurements, the average attenuation for a cylinder at the center of the sample with a diameter that was three-quarters of the total diameter was analyzed to avoid the worst cupping artifacts. The relationship between the average measured linear attenuation of the central cylinder and the K$_2$HPO$_4$ concentration appeared to be non-linear for the non-corrected scans (Fig. 1D). The obtained linear attenuation values were less than the theoretical ones and the difference between the

Developing mandibles

Two porcine mandibles (sus domesticus) of different developmental age were used as an example of the progress of mineralization in the ossification process. One was from a female fetus (gestation age between 55 and 60 days and crown-rump length (CRL) of 124 mm). The other was from a newborn female pig (CRL of 392 mm). The mandibles were removed by dissection and cut in half at the symphyseal region. They were stored in a 4% formaldehyde solution at ambient temperature. Prior to scanning, the hemimandibles (right side) were mounted in a cylindrical specimen holder and secured with synthetic foam. The holders were filled with water until the specimens were completely submerged.

The specimens were scanned completely with a $10 \times 10 \times 10 \mu\text{m}^3$ and $30 \times 30 \times 30 \mu\text{m}^3$ resolution for the fetal and newborn mandible, respectively. The hemimandible of the newborn pig was rescanned with a resolution of $10 \times 10 \times 10 \mu\text{m}^3$ after it had been cut into a number of sections. The bone scans were performed with low energy (45 kV/24 keV).
two increased with the \( \text{K}_2\text{HPO}_4 \) concentration. The beam-hardening correction algorithm, however, improved the relationship between linear attenuation and \( \text{K}_2\text{HPO}_4 \) concentration considerably (Fig. 1D). Therefore, all subsequent reference and bone measurements were performed with implementation of this beam-hardening correction algorithm.

The results of the reference measurements are depicted in Fig. 2. For small specimen diameters the resulting linear attenuation coefficients were larger than for larger specimens. Furthermore, for scans with low energy (45 kV/24 keV), the resulting linear attenuation coefficients were larger than for scans performed with high energy (70 kV/31 keV), which was consistent with theoretical linear attenuation values from the NIST database. The measured attenuation in the case of the lower concentrations of the \( \text{K}_2\text{HPO}_4 \) solutions lay above the theoretical values, whereas with higher concentrations the linear attenuation coefficients lay beneath the theoretical value. The variation in current had a negligible influence on the measured attenuation values (data not shown).

**Accuracy**

In order to determine the accuracy of the measured mineralization density, the measured linear attenuation coefficients were compared with the theoretical ones for the concentration range in every energy-sample diameter combination. With the exception of the scans of the smallest samples with high energy settings, the average relative errors over the range of concentrations were around 10% or less (Fig. 3A). Furthermore, scans performed with low energy settings deviated less from the theoretically determined linear attenuation values than those performed with high energy, especially where smaller specimens were concerned.

An overview of the specific error as a function of the concentration is shown in Fig. 3B. This figure shows the average error over the complete range of concentrations for a scan with a sample diameter of 20 mm, which were scanned using low energy (24 keV). The smallest errors occurred in the concentration region between 400 and 800 mg/cm\(^3\). The minimal error shifted to higher mineralization density values with decreasing specimen dimensions (data not shown). The resolution of the scan had no influence on the accuracy of the measured attenuation coefficients (data not shown).

**Developing mandibles**

Comparison of the reconstructions of the two mandibular specimens revealed considerable morphological differences between the two developmental stages (Figs. 4 and 5). The mandible of the fetal pig showed no solid cortical bone, in contrast to the mandible of the newborn. In the newborn mandible the bone in the coronoid and condylar processes and the symphyseal region was clearly visible.

The degree of mineralization appeared distinctly higher in all regions of the mandible of the newborn pig (Table 1). In both the fetal and newborn mandible a distinct difference in the degree of mineralization between the different regions could be distinguished (Table 1). In both specimens, the corpus was the region that showed the highest degree of mineralization. The lowest degrees of mineralization were observed in the condylar head and the mandibular angle. Also, at the trabecular level, mineral density distribution differences within trabeculae were observed between the fetal and the newborn specimen (Fig. 6). The trabeculae showed a higher degree of mineralization in their centers.

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Fig. 2. Calibration lines of different combinations of sample dimensions and energy settings for the relationship between sample concentration and the linear attenuation coefficient. Solid line, theoretical relationship; dotted line, specimen diameter 12 mm; dash-dot line, specimen diameter 20 mm; dashed line, specimen diameter 36 mm.
than near the edges. Besides the overall thickening of the trabeculae, more regions of higher degrees of mineralization were recognized in the newborn specimen.

**Discussion**

This study deals with quantification of the degree of mineralization in developing bone using a commercially available microCT device equipped with a polychromatic X-ray source. It was hypothesized that the measured attenuation profiles in developing bone were accurate enough to make quantitative comparisons despite the beam-hardening effect, which is a common artifact when using polychromatic X-ray sources.

It appeared that the absorption of X-rays by various solutions of K$_2$HPO$_4$ was considerably underestimated due to beam-hardening. This could be largely corrected by applying the correction algorithm with which the microCT system was equipped. However, the remaining beam-hardening effect still had some influence. Subsequent reference measurements, for instance, showed that the attenuation values in reference specimens with smaller diameters were higher than for larger specimens, although they were of the same concentration K$_2$HPO$_4$ (Fig. 2). This can be explained by the fact that the soft radiation was absorbed equally at the surfaces of both the small and large specimens. As the volume/surface ratio increases with the diameter, the attenuation was lower in a larger area. Averaging the attenuation over a cylindrical section in the center of a reference specimen thus led to a greater accuracy of the attenuation values in reference measurements with a larger diameter. The high measured attenuation values found in small diameter tubes is probably due to an overcorrection of the beam-hardening correction algorithm. The fact that the measured reference curves lay above the theoretical curve in all cases, when low K$_2$HPO$_4$ concentrations were concerned (Fig. 2), has to be attributed to the applied beam-hardening correction function. Furthermore, the theoretical attenuation values were obtained by assuming the average effective
energy as the representative of the polychromatic radiation spectrum.

As mentioned above, the beam-hardening correction function did not have a completely linearizing effect on the measured attenuation curve, and deviations from the theoretical curve remained. These errors remained substantial, especially where higher bone mineral densities were concerned. As the degree of mineralization in adult bone often exceeds 1250 mg/cm$^3$ (15), errors larger than 20% can be expected despite application of the correction algorithm. Moreover, specimens with high mineralization densities may be impenetrable to low energy radiation and thus require high energy application, which in turn causes a further decrease in accuracy. For fetal bone specimens, however, the present correction function proved to be sufficient to obtain adequate results. Average deviation from the theoretical attenuation profile was below 10% for all sample dimensions and energy-current settings, with the exception of the high energy measurements of the smallest sample (Fig. 3A). Errors were even smaller in the density range between 300 and 600 mg/cm$^3$ (lower than 6%). This accuracy is comparable to that obtained with synchrotron radiation, which reaches an average deviation from theory of less than 6% irrespective of the degree of mineralization (16). Still, extrapolation of these results to measurements of the degree of mineralization of bone has to be performed with care. The present results were obtained with homogeneous solutions of K$_2$HPO$_4$. Bone, however, is a heterogeneous material and radiation absorption in specific parts of the bone may influence the absorption of radiation in an adjacent region of bone. Furthermore, the high degrees of

<table>
<thead>
<tr>
<th>Region</th>
<th>Fetal degree of mineralization (mg/cm$^3$)</th>
<th>Estimated error* (mg/cm$^3$)</th>
<th>Newborn degree of mineralization (mg/cm$^3$)</th>
<th>Estimated error* (mg/cm$^3$)</th>
</tr>
</thead>
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<tr>
<td>Symphysis</td>
<td>382</td>
<td>17</td>
<td>751</td>
<td>40</td>
</tr>
<tr>
<td>Corpus</td>
<td>650</td>
<td>23</td>
<td>985</td>
<td>89</td>
</tr>
<tr>
<td>Angle</td>
<td>360</td>
<td>18</td>
<td>651</td>
<td>21</td>
</tr>
<tr>
<td>Ramus</td>
<td>586</td>
<td>5</td>
<td>803</td>
<td>47</td>
</tr>
<tr>
<td>Coronoid process</td>
<td>616</td>
<td>7</td>
<td>843</td>
<td>55</td>
</tr>
<tr>
<td>Condylar neck</td>
<td>550</td>
<td>3</td>
<td>642</td>
<td>9</td>
</tr>
<tr>
<td>Condylar head</td>
<td>279</td>
<td>20</td>
<td>580</td>
<td>12</td>
</tr>
</tbody>
</table>

*Error of the degree of mineralization of bone was estimated according to the accuracy of the reference measurements.
mineralization of teeth that are present in the later developmental stages have an unknown influence on the measured degree of mineralization of adjacent bone and caused some streaking. Moreover, the presence of soft tissues could have influenced the attenuation of radiation. As this has only a minor contribution to the absorption of rays (approximately 7%), it was not elaborated in this study (21).

A wide variety in degree of mineralization as expected in the developing mandible could indeed be retrieved from the attenuation maps obtained from the two porcine mandibles (Figs. 4 and 5 and Table 1). They reflect a normal mandibular development. At locations where the ossification process of the mandible starts [the corpus (28)], the obtained attenuation was relatively large, whereas at sites that ossify much later – for instance the symphysis and condylar head, which are both involved in endochondral ossification (13, 18) – it remained relatively low. Furthermore, the degree of mineralization at the mandibular angle was relatively low. This could be related to the backward growth of the ramus and angle, where new, not fully mineralized bone is constantly added to the posterior border. As this process continues after birth, the degree of mineralization in the mandibular angle of the newborn specimen was relatively low. Also, in all other regions, the degree of mineralization had increased, but the relatively small increase of the degree of mineralization of the condylar neck is presumably caused by the continuous upward growth of the condyle.

There were also differences observed in bone mineral distribution at the trabecular level. With age, the overall thickness of the trabeculae increased and higher degrees of mineralization were found in the centers of the trabeculae (Fig. 6). The observed lower mineral density at the surfaces of the trabeculae could be caused partially by a volume effect, i.e. voxels at the surfaces may be partly filled with bone. The computed degree of mineralization for such a voxel could therefore be reduced. This effect can only be present in a shell with a thickness of 10 μm (the resolution). As the trabeculae have a thickness of 70–100 μm, most of the observed mineralization differences have to be attributed to real differences. This suggests that new unmineralized bone tissue is laid down at the surfaces of the trabeculae and progressively mineralizes, leading to more mature and thus more mineralized tissue in their centers.

It can be concluded from this study that the applied microCT system is adequate to quantify the degree of mineralization in developing bone. It provides a means by which to simultaneously investigate the three-dimensional structure and mineral density distribution of developing bone. It was proved adequate to assess the relative complex timing of the mineralization process present in the mandible in a non-destructive manner. The resolution is high enough to observe the architecture and the degree of mineralization on a trabecular level. In adult bone, beam-hardening presumably disturbs the quantification of the degree of mineralization unacceptably, as errors larger than 20% can be expected.

The present method can provide a better understanding of the development of the relationship between the degree of mineralization and architectural parameters such as bone volume fraction, trabecular number, trabecular thickness, and anisotropy. The combined investigation of the degree of mineralization and the architecture could provide a better comprehension of the mechanical properties of trabecular bone. The use of this method might be applied in the future to other parts of the skeleton to enrich the basic embryological information. Furthermore, the method might be useful for evaluating the influence of genetic diseases, pathogenic drugs, or other noxious environmental conditions on the fetal development of bone.

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