Demineralization of cortical bone for improvement of Charpy impact fracture characteristics

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Abstract
Bone tissue is a composite structure of apatite particles distributed in collagen fibril matrix on a nano-size scale. Mechanical properties of cortical bone are determined by the apatite-collagen composition. The amount and specific orientation of apatite crystals strongly affect the mechanical properties of macroscopic bone tissue such as anisotropic elastic modulus and fracture toughness. The progression of mineralization with tissue aging results in a reduction of fracture toughness due to embrittlement of tissue caused by excessive increase of apatite density. This study focused on the change of impact fracture characteristics of cortical bone tissue with reduction of apatite concentration (demineralization). A compact-sized impact loading device for Charpy tests was developed to measure the absorbed energy for impact fracture of bone specimens in the 0.5 and 1.0 J input energy range. Cortical bone specimens of 3 × 3 × 30 mm were prepared from shaft of bovine femurs. Differences relating to the anisotropy in axial and circumferential direction of the femur were observed in the absorbed energy values. The values of the axial specimens were greater than the circumferential specimens. Axial bone specimens were demineralized in ethylenediaminetetraacetic acid (EDTA) solution at 5°C. The demineralization progressed slowly from surfaces of the specimen. The 24-hour demineralization created a collagen layer at the surface of specimen and the demineralized specimen showed higher absorbed energy than unprocessed specimens. The absorbed energy in defective specimens with a square shaped small slit of 0.5 × 0.5 mm increased after local demineralization process. The time for effective demineralization could be reduced to 2 hours in the case of 37°C condition. The demineralization process improved the fracture characteristics of both intact and defective cortical bone tissue.

Key words: Bone tissue, Fracture, Charpy impact test, Apatite, Demineralization

1. Introduction
The likelihood of living a long life of more than one hundred years has been increased by the great innovations of medical technology in recent years. However, maintaining the ability to walk by own legs over the long lifetime has never been ensured. The risk of bone fracture increases steadily with age due to brittleness of bone structures as typified by osteoporosis. Many kinds of tissues may be regenerated and replaced by stem cells or artificial organs in the near future. Improvement of bone structure also may be performed in the tissue engineering. However the application of tissue engineering for repair and reconstruction of the bone tissue remains to be a challenge. Although bone remodeling usually occurs in our internal structure, the cycle requires a long period of several months. Bone regeneration also depends on the time scale of the cycle. Living bone tissue has a structural adaptation function responding to external loads (Currey, 2003). If people stay in bed for the long time waiting for the regeneration process after bone fracture, it induces bone weakening caused by lack of stress stimulation. Thus the improvement of bone tissue should be performed in a rapid process. Mechanical properties of bone tissue are strongly affected by hierarchal structure formed by oriented materials and their combination on various scales (Fratzl and Weinkamer, 2007). This study focused on the structural interaction of microscopic components of bone tissue to discuss how to improve the fracture characteristics.
Bone tissue is a composite material consisting of hydroxyapatite-like mineral particles and proteins principally involved in type I collagen production on a nano-size scale. The mineral, protein, and water content of bone is 65, 25, and 10 wt.%, respectively (Olszta et al., 2007). The mechanical properties of bone tissue were well investigated and focused on the characteristics of the mineral-collagen scale. The crystal structure of apatite is inhomogeneous (Raquel, 1981, Matsushima et al., 1986) and particle sizes are varied in bone regeneration processes, (Liu et al., 2010). The microscopic structure of the apatite and collagen network is an indicator of bone quality, affecting the mechanical properties of aged bone. The crystal orientation of apatite in bone tissue was used to evaluate the bone quality (Nakano et al., 2002, 2012). The alignment of apatite crystals determines the anisotropy of bone tissue (Sasaki et al., 1989, Sasaki and Sudo, 1997, Giri et al., 2009), and the highest elastic modulus is observed along the axial direction in the shaft of long bones (Yamamoto et al., 2012). The strain behavior of apatite crystals in bone tissue has been investigated using X-ray diffraction (Gupta et al., 2006, Fujisaki et al., 2006, Almer et al., 2007, Giri et al., 2012). It was confirmed that the hardness of apatite components and strain sharing ratio of apatite phases determine the elastic modulus of macroscopic bone tissue (Fujisaki and Tadano, 2007). The change in apatite density in cortical bone strongly affects the rigidity because the elastic modulus of apatite is more than one hundred times greater than that of the collagen matrix (Zamiri and De, 2011). The reduction of elastic modulus of cortical bone in demineralization processes was confirmed in stress-strain measurements of static tensile loading (Todoh et al., 2009). However, demineralized collagen fibers give high bendability of the structure. An appropriate balance between collagen and apatite content is essential for maintaining the desired material properties. Hence, excessive mineralization due to tissue aging increases brittleness and reduces toughness of the tissue (Currey et al., 1996, Currey, 2004, Roschger et al., 2008). In this study, demineralization is used as a chemical treatment of bone tissue in order to change the impact fracture characteristics. A compact-sized impact loading device for Charpy-type tests is developed to evaluate the impact fracture characteristics of cortical bone specimens. The effect of apatite eliminations on the impact behavior is investigated in the fracture tests and observations of demineralized tissue. In addition, bone structural damage causes a stress concentration and brittle fracture of the structure. We propose a method for improving the impact fracture characteristics of bone tissue with the structural defect by means of local demineralization processes.

2. Materials and Methods

2.1 Charpy impact tests

A Charpy-type impact loading device was designed taking into considerations of the range of absorption energy during fracture of cortical bone specimens. An impact load was applied under three-point bending condition with two support points at a distance of 24 mm; 12 mm on either side of the impact point. The input load was applied at the center of the longitudinal axis of the bone specimens. Figure 1 shows the impact loading device developed here. The input energy of the impact tests was set to either 1.0 or 0.5 J by changing the arm part of the impact hammer. The specifications of the testing device are listed in Table 1. The impact rates of both conditions were designed to be around 3 m/s. The absorption energy was measured as the reduction of potential energy during the hammer swing before and after specimen destruction. The impact fracture toughness of bone tissue relates to the value of absorption energy. The angle change of the hammer swing was tracked by observing the rotation indicator by means of high-speed camera with 200 fps. The absorbed energy, $W_C$, was calculated by using Eq. (1),

$$W_C = M_H (\cos \alpha_R - \cos \alpha_0) - W_f$$

(1)

Where, $M_H$ is the hammer moment determined by hammer weight and distance from the center of rotation to the weighted center of the hammer arm. The angles $\alpha_0$ and $\alpha_R$ are measured at the initial and the end positions of the hammer swing, respectively. The friction loss $W_f$ was measured without specimen destruction, and the loss was below 1° in the swing system.
2.2 Sample preparation

Cortical bone samples were prepared from bovine femurs extracted from mature cows of 22 month old (sample 1) and 28 month old (sample 2). Column-shaped specimens 3 × 3 × 30 mm in size were formed using a low speed wheel saw (Model 650, South Bay Technology Inc.) under wet conditions with physiological saline applied to prevent sample desiccation and heat generation. Impact loading tests were conducted under the three point bending condition. The long axes of the specimens were selected along with each direction corresponding to the bone axial (specimen A) and circumferential (specimen C) direction, as shown in Fig. 2. There were obvious differences in mechanical properties between samples A and C because the cortical bone at mid-shaft of long bone shows strong anisotropy (Fujisaki et al., 2006). The difference of these two specimens can be mainly used to confirm the performance of the impact testing device. The acrylic specimens in same size were also prepared for reference materials without individual differences. The specimen A was used to investigate the fracture characteristics considering with bending situations of long bones and the effect of chemical treatments for demineralization on the bending fracture characteristics. Defective specimens were created using an additional removal machining process. A small size 0.5 × 0.5 mm square-shaped slit, as shown in Fig. 2, was created using the low speed wheel saw at the center of the surface experiencing tensile strain under the three-point bending, which was on the opposite side of the impact point as shown in Fig. 1.

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Input energy [J]</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hammer weight [kg]</td>
<td>0.512</td>
<td>0.752</td>
</tr>
<tr>
<td>Distance of weight center [mm]</td>
<td>82.9</td>
<td>98.0</td>
</tr>
<tr>
<td>Hammer moment [Nm]</td>
<td>0.416</td>
<td>0.723</td>
</tr>
<tr>
<td>Initial angle [deg]</td>
<td>103</td>
<td>112</td>
</tr>
<tr>
<td>Impact rate [m/s]</td>
<td>3.03</td>
<td>2.94</td>
</tr>
</tbody>
</table>

Fig. 1 A compact-sized Charpy-type loading device. The input energy can be selected at 0.5 or 1.0 J by changing the hammer arm. This picture shows the device having 0.5 J arm.

![Fig. 1](https://example.com/fig1.png)

Fig. 2 Cortical bone specimens cut out from a bovine femur. A square shaped small slit was added to the surface of specimen A. Tensile stress applied to the defect area in three point bending condition in Charpy impact test.

![Fig. 2](https://example.com/fig2.png)
2.3 Chemical treatment

Bone demineralization was performed in 10% (w/v) ethylenediaminetetraacetic acid (EDTA) - water solution in a 300 ml vessel. Two types of demineralization conditions were used in this experiment. In the first condition, bone specimens were dipped in EDTA solution at 5°C under agitating with a magnetic stirrer for the duration of treatment. The chemical reaction progressed gradually for several days. The second condition utilized the EDTA solution at a physiological temperature of 37°C which was expected to reduce the treatment time and to confirm the clinical relevancies. The EDTA was saturated in both conditions. In the case of defective bone study, the specimens were masked to limit the treatment area near the slit and then placed in the EDTA solution with agitating. The mechanical properties of intact, defected and local demineralized bone specimens were measured in both the static and the impact loading tests.

3. Results

3.1 Evaluation of impact testing

Charpy impact loading tests were conducted for specimens A and C without notch cut out from sample 1. The fractures occurred at the center of the specimen during the three-point bending in the impact test. The direction of crack propagation on the fractured surface was irregular in the specimen A and straight in the specimen C. Figure 3 shows the absorbed energy measured in each specimen under the 1.0 J input energy condition in Charpy impact loading test. The values (mean ± S.D. (n=5)) were 55.3 ± 3.0 mJ in the axial (specimen A), 20.0 ± 3.0 mJ in the circumferential (specimen C) specimens, and 61.2 ± 2.8 mJ in the referenced acrylic specimens. The specimen A had significantly larger absorption energy than the specimen C. The repeatable accuracy of the experiment using this device can be evaluated from the value of standard deviation of acrylic results which is thought to have little individual difference. Although the bone specimens usually have individual differences even if the specimens are taken from same bovine femur, the standard deviation values of the both bone specimens were almost same comparing with the acrylic result in this experiment.

![Fig. 3](image)

3.2 Demineralization process

The bone axial specimens (specimen A) were chemically treated. Demineralization gradually progressed from the surface of the specimen in the 5°C temperature condition. The cross-sectional images of the 24 h treated are shown in Fig. 4(a). The demineralization layer was created at the surface of specimen. The thickness of the obvious demineralized area which was recognized in the optical image was 90 μm after 24 h, and then up to 180 μm after 48 h.
in the EDTA solution. The demineralization rate slowed after 48 h. The complete demineralization required a longer period of more than two weeks in the 5°C temperature condition. The same demineralization thickness of around 90 μm could be obtained within 2 h in the 37°C temperature condition, as shown in Fig. 4(b). The demineralization was also progressed from the surface of the specimen in these conditions. The treatment area could be limited by using vinyl tape masks. An unmasked area was located at the center of specimen with 4 mm width (BS) which was demineralized at the both sides of tensile and compressive deformation in bending. And an unmasked area with 2 mm only on the tensile side at the center of specimen (TS) was used for performing the locally focused demineralization.

3.3 Absorbed energy in demineralized specimen

Figure 5 shows the absorbed energy of specimen A in sample 1 without defect (Sample 1_intact) prepared under different demineralization treatment times of 12, 24, 48 and 72 h in the 5°C temperature condition. The experiments were conducted under an input energy of 1.0 J. The absorbed energy increased after 24 h demineralization. Although the prolonged treatments of more than 48 h reduced the absorbed energy, indestructible areas were observed at the demineralized layer on the compressive surface of specimens. The specimen that was demineralized for more than one month had high bendability with low elastic resistances and could not break under the impact loading condition.

The experiments for defective specimens were conducted under 0.5 J input energy. The masks with 4 mm unmasked at the center of specimens were used to limit the treatment area. The addition of a slit-shaped defect significantly reduces the impact fracture strength because of both the stress concentration around structural defect and the reduction of cross sectional area at the impact point. The time-dependent effects of demineralization at 5°C on the absorbed energy measured in both samples are shown in Fig. 5. And there were individual differences between the specimens taken from sample 1 and sample 2. The absorbed energy values increased even after 72 h treatment compared with untreated state in both sample.

Figure 6 shows the absorbed energy of demineralized specimens (sample 2) with the structural defect and treated at 37°C for 2 h. Two types of masks were used here, expressed in Fig. 6, one with a 4 mm exposed area including both the tensile and compression sides of specimens (BS) and another one with a 2 mm exposed area on the tensile (defected) side only (TS). The values (mean ± S.D. (n=5)) were 8.4 ± 4.0 mJ in initial condition (W/O), 25.5 ± 6.4 mJ in BS, and 21.2 ± 5.0 mJ in TS specimens. The significant differences (P<0.01) in two-sample unequal variance t-test between W/O and BS, W/O and TS were obtained with P-values (one-sided) of 0.0007, 0.001, respectively. There were obvious increases in the absorbed energy values after demineralization under both the BS and TS conditions. The results showed that the demineralization improved the impact fracture resistance even if the treatment was limited to the local area in the immediate vicinity of small defect (TS).

The load-deflection curve with 0.1 mm/min loading rate of typical bone specimens were shown in Fig. 7. These static loading behaviors were obtained from the specimen without defect (intact_0h) and the defected specimens before treatment (defect_0h) and after treatment (TS_2h). Both the maximum load and the maximum deflection obviously decreased by creating the small slit. The fracture behaviors were brittleness in the case of before treatment. Although the elastic modulus decreased in the demineralized specimen case, static bendability was higher than the before treatment.
Figure 8 shows the scanning electron microscope (SEM) images of fracture surfaces of impact loading and static loading specimens with and without demineralization treatment in the TS condition. The demineralize tissue effects were confirmed at the edge of defected area, as shown in (b) and (d). The fracture surfaces showed distinguishing pattern in each situation. The fracture surfaces in impact fracture cases were smoother than in static cases. The osteon patterns with Haversian canals were recognized in the demineralization case under impact loading (b).

![Graph showing absorbed energy vs demineralization time](image)

**Fig. 5** The absorbed energy of specimens prepared in different demineralization treatment times. “Sample1_intact” means specimen A taken from sample 1 without defect measured at 1.0 J input energy. “Sample1_defect” and “Sample2_defect” mean specimen A with a defect taken from sample 1 and 2, respectively, measured at 0.5 J input energy.

![Absorbed energy graph](image)

**Fig. 6** Absorbed energy (mean ± S.D. (n=5)) of defective specimens (Sample 2, specimen A) after 2 h of demineralization under 37°C with different masking conditions. “BS” is 4 mm of unmasked width on both the compressive and tensile strain sides of the specimen, “TS” is 2 mm unmasked width on the tensile loading side of the specimen.

![Load-deflection curve graph](image)

**Fig. 7** Static bending behavior of typical specimens (Sample 2, specimen A) of intact and defected with and without demineralization treatment. The load-deflection curves were measured in three points bending as the same geometry of the impact loading test.
4. Discussion

Impact fracture characteristics of cortical bone specimens with a slit shaped defect were explored in this study. We used a compact sized Charpy type loading device which can work with the small impact energy of 0.5 J, and the differences of absorption energy of each specimen were measured. Both the defected specimens based on specimen A and the intact specimen C absorbed the energy only less than 25 mJ during the impact fractures, expressed in Fig. 3 and 6. Reliability of the value could be confirmed by considering with the static fracture absorption energy calculated from the areas of load-deflection curves for specimen A with defect. However this input energy level is too small to discuss the detail process of deformation behavior at the impact moment. A more accurate impact test with small input energy is required for analyzing the function of demineralized collagen layer. The fracture surfaces inclined with the perpendicular direction of bending axis. And non-smooth fracture surfaces were obtained in bone axial specimens because of bone axial oriented osteon structures. Osteon structures of bovine femoral bone often show plexiform patterns. There is a possibility that the crack propagation into the bone tissue is to be complex behaviors compared with human bone constructed with circular osteon structures. The cross sectional areas directly correspond to the absorption energy especially in bone axial and circumferential without slit cases.

Fracture toughness of bone is greatly affected by water content, amount of tissue, porosity, osteon generation, and mineral content (Aerssens et al., 1998). The mineral content of bone can be manipulated by using chemical treatments. The demineralization by means of chemical treatment usually progresses from the outer surface of the bone, which was observed in this study. The surface strain has the maximum value during bending of the cortical shell. The stress reduction based on elimination of stress-strain concentrations was resulted in the demineralization process. Although the obvious demineralized area was less than 100 μm in the suitable condition here, the absorption energy increased in both intact and defective specimens. The fracture surface of demineralized specimen was extensively different from the fracture surface of untreated specimen. The osteon patterns with Haversian canals were observed in the SEM image of fracture surface of the demineralized specimen as shown in Fig. 8. Thus demineralization effects from the surface might be progressed mainly though the boundary of osteons. The fracture surfaces are also depending on the strain rate in bending for bone axial specimen (Zimmermann et al., 2014). Fracture surface observations are important for evaluation of the effectiveness of demineralization treatments in our future works.

The demineralization process is often used to observe the anisotropic structure of collagen matrix in bone tissue (Novitskaya et al., 2011). For example, microscopic observation of the apatite structure and the collagen orientations in bone tissue can be conducted in deproteinization and demineralization treatments, respectively. As expected, the mechanical properties can be strategically changed as a result of these usual treatment processes. We insist that the widely used demineralization technique is available not only to analyze the bone structure but also to improve the impact fracture characteristics of bone. The bending load usually acts to the specific part such as femoral neck, mid part of long bone and the stress concentration often causes bone fracture. Thus, the locally-controlled demineralization of micro-scale area is expected to reduce the risk of bone fracture without reduction of macroscopic rigidity of the area.

Fig. 8 SEM images of fracture surfaces of impact loading specimens (a) without treatment and (b) with 2 h treatment in tensile side (TS), and static loading specimens (c) without treatment and (d) with 2 h treatment in TS.
this study, the demineralization process was controlled by EDTA solution. The EDTA treatment is used for chemical treatment of extracted human bone tissue. If we use this like demineralization technique for reducing the risk of living bone, it is required to investigate the damage of surrounded living tissue and how to supply and restrict the diffusion of solution in vivo situations.

This study focused on only the apatite phase of bone tissue by decreasing apatite content via demineralization in order to improve the fracture characteristics. Aged bone has not only excessive mineralization tissue but also damaged collagen fibers (Manilay et al., 2013). The crosslinking of collagen fibrils is also a cause of bone brittleness. Adjustments to both the apatite composition and collagen flexibility are required for repairing the aged bone tissue.

5. Conclusion

The impact fracture characteristics of bovine cortical bone specimens were evaluated using Charpy-type impact loading tests. The anisotropic fracture characteristics measured as differences of absorbed energy values were clearly obtained in this system. These values were higher in the axial bone specimens than in the circumferential specimens. The demineralization using EDTA solution progressed from the surface of bone specimen and demineralized layer was created in this process. The absorbed energy in the impact fracture tests for both the intact and the defective bone specimens increased in the specific demineralization condition. The demineralization in the immediate vicinity of the defective area was enough to improve the impact fracture characteristics of cortical bone tissue.

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References


