Influence of short-term fixation with mixed formalin or ethanol solution on the mechanical properties of human cortical bone

Abstract: Bone specimens obtained for biomechanical experiments are fresh-frozen for storage to slow down tissue degradation and autolysis in long-term storage. Alternatively, due to infectious risks related to the fresh tissues, fixative agents are commonly used. However, fixatives will likely change the mechanical properties of bone. Existing studies on this issue gave controversial results that are hardly comparable due to a variety of measurement approaches. For this reason, the influence of ethanol and a formalin-based fixative agent was evaluated on the mechanical properties of human cortical bone specimens by means of four-point-bending tests. 127 prismatic specimens with rectangular cross sections (2.5 x 2.5 x 20 mm³) were obtained from different regions of two fresh human femora (medial, lateral, dorsal, ventral). Specimens were either fixed in ethanol or in a mixed formalin solution or frozen following a given scheme. After two weeks of storage the samples were re-hydrated in isotonic saline and subsequently tested mechanically. The elastic bending modulus and ultimate bending strength were computed considering the actual dimensions of each specimen. For statistical analysis a one-way-ANOVA and an LSD post-hoc-test were performed. For ultimate bending strength no significant differences due to formalin or ethanol fixation, as compared to unfixed-fresh bone specimens could be found. And only for few cases significant differences in elastic bending modulus were observed when the two bones were evaluated separately. Since more differences of significant level due to the anatomical region of the samples were determined, the original location seems to have more influence on the evaluated mechanical properties than the method of (chemical) fixation. Consequently, ethanol and the mixed formalin solution can be recommended as a fixation agent for samples in biomechanical testing, if these samples are rinsed in isotonic saline prior to static mechanical testing.

Keywords: human cortical bone; mechanical properties; fixation; ethanol; formalin; 4-point-bending

Introduction

In biomechanics and biomedical engineering, mechanical tests and in-vitro experiments are required to obtain material properties or to validate numerical simulations. These experiments should be executed in an in-vivo-like environment. Bones and derived specimens are often stored in a frozen condition as a gold standard. This procedure has been proven to alter most of the mechanical properties of bony material to minor extent [1–3]. However, when dealing with fresh-frozen specimens, there is a potential infection risk for researchers being exposed to the tissues. Furthermore, it is nearly impossible to perform long-term tests with unfixed specimens due to the ongoing processes of tissue degradation and autolysis [4].

In order to minimize infectious risks and to enhance the durability of biological tissues, specimens are often fixed, using different chemical agents. There have been several attempts to investigate the influence of said fixatives on the mechanical properties of bone. However, there is still a lack of distinct findings due to the miscellaneous conditions under which most studies have been performed. Some research groups used human bones [1, 5, 6] while others carried out their tests with faunal bones [3, 6–10]. Furthermore either entire bones [3, 8, 10, 11] or machined specimens with uniform geometrical shape [1, 5–
were used in varying testing setups, including three- and four-point-bending, compression, tension and torsion tests. Accordingly, the results and conclusions drawn from these studies differ to large extent. Some authors reported substantial influences of fixation on the mechanical properties \[9, 11\] while others identified only slight effects \[1, 5–8, 10, 12, 13\] or even hardly any influence at all \[3, 14\].

The following experimental study was conducted to evaluate the influence of two different fixative agents on the mechanical properties of human femoral cortical bone. We hypothesize that there are differences between chemically unfixed specimens and those treated with ethanol or a mixed formalin solution. Our second hypothesis is that mechanical properties of bone are more dependent on the anatomical region than on the method of fixation.

## 2 Material and methods

### 2.1 Sample preparation and treatment

Bone samples were obtained from two right-sided human femora (F1/2) in the fresh condition. While alive, the body donors gave their informed consent to the donation of their bodies for research purposes after their passing. The femora were pre-cooled and subsequently shock-frozen at 80 °C for storage. Bone samples were taken from the ventral, dorsal, medial and lateral compacta with a diamond blade band saw BS270-S (DRAMET Drahth und Metallbau GmbH, Kleinmaischeid, Germany). The dimensions of each sample were 2.5 by 2.5 mm and 20 mm along the femoral shaft axis. The resulting specimens were again stored at 80 °C and numbered in a sequence from proximal to distal.

Depending on the number of specimens obtained from every single (long) beam, the samples were fixed with chemical agents following a random scheme depicted in Figure 1. to ensure a proper statistical distribution. Two fixation fluids were used: technical ethanol (96 % by mass, Brenntag GmbH, Mühlheim, Germany) or a mixed fixation solution containing formalin, ethanol, distilled water, glycerin, thymol, and salicylic acid at given volumes of 5.5%, 60.7%, 24.8%, 8.3%, 0.5%, 0.4% and 0.3%, respectively (“Hauslösung” supplied by the Department of Anatomy of the University of Rostock). The corresponding 2 ml tubes were filled with the fixation fluid covering the entire bone specimen and a volume ratio between fixative and specimen of at least 10:1. The chemically fixed samples were stored at room temperature for 14 days, while the unfixed-fresh ones were stored in shock-frozen condition. Table 1 shows the distribution of fixed specimens for each bone. The frozen unfixed specimens were thawed and both the unfixed and the fixed specimens were rinsed in isotonic sodium chloride solution for at least 30 minutes to minimize a possible influence of dehydration \[15\] or to remove the fixatives prior to material testing. The intrinsic height and width of every single sample were obtained using a caliper.

<table>
<thead>
<tr>
<th>bone</th>
<th>number obtained</th>
<th>ethanol</th>
<th>mixed fixation</th>
<th>unfixed-fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>70</td>
<td>24</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>F2</td>
<td>57</td>
<td>19</td>
<td>15</td>
<td>23</td>
</tr>
</tbody>
</table>

### 2.2 Mechanical testing

All specimens underwent a four-point-bending test conducted in an electromechanical testing machine Zwick-Line Z1.0 and a force sensor XforceP 1kN (both Zwick GmbH & Co. KG, Ulm, Germany; precision 4 N). The width S between the supports was set to 16 mm while the loading span L was 6 mm (Figure 2). After a dwell of 2 s at a preload of 2 N the sample was loaded at a crosshead speed of 1 mm/min until material failure occurred, indicated by a visible breaking of the sample into separate pieces or by a slow but steady decrease of force after a distinct maximum value.

The data were obtained and analyzed with testXpert II software (V3.4, Zwick GmbH & Co. KG, Ulm Germany).
Further investigation of the data was processed with Excel 2007 (Microsoft Corporation, Redmond, WA, USA). For each specimen the load-displacement-diagram was analyzed and the corresponding linear slope ($\Delta F/\Delta w$) was determined for the region of elastic material behavior. The regression should end up with a coefficient of determination higher than 0.995. Ultimate stress and elastic bending modulus were calculated based on the sample width $b$, height $h$ and lever arm $a = 5$ mm by:

$$\sigma_{\text{max}} = \frac{3 \cdot F \cdot a}{b \cdot h^2}$$

$$E = 5.6 \cdot \frac{\Delta F}{\Delta w} \cdot \frac{a^3}{1000 \cdot b \cdot h^3}$$

Statistical analyses were performed using SPSS (V20, IBM Corp., Armonk, USA) via one-way-ANOVA followed by a LSD post-hoc-test. The level of significance was set to $p < 0.050$.

### 3 Results

The evaluation of all tested specimens without consideration of the anatomical region revealed values for ultimate bending strength of 175.0 ± 43.0 MPa (mean ± standard deviation) for (et), 175.1 ± 41.6 MPa for (mf) and 171.7 ± 40.0 MPa for (uf). The elastic bending modulus was determined as 8.7 ± 1.8 GPa (et), 9.2 ± 1.8 GPa (mf) and 9.1 ± 1.8 GPa (uf), respectively. Neither of them showed any differences of significant level ($p > 0.05$). Distinguishing between the two investigated bones led to similar results.

Evaluating a possible influence of the fixation method on the mechanical properties for each anatomical region separately revealed significant differences in elastic bending modulus between ethanol and mixed formalin ($p = 0.047$) and between ethanol and unfixed-fresh samples ($p = 0.038$) for the ventral region of F1 and between ethanol and unixed-fresh ($p = 0.035$) for the lateral region of F2. Summarizing all samples of both bones showed no significant differences depending on the fixation method.

The evaluation of regional dependencies of mechanical properties within groups of equally treated specimens showed differences of significant level in ultimate bending strength between dorsal and ventral samples fixed with ethanol ($p = 0.017$) as well as with mixed formalin ($p = 0.020$) for F1. Furthermore, the ultimate bending strength differed significantly for unfixed-fresh samples from the medial and the lateral regions ($p = 0.014$) and from the lateral and the ventral regions ($p = 0.041$) of F2. When all samples were put together, a significant difference in ultimate bending strength was found for unfixed-fresh bone from the medial and the lateral regions ($p = 0.009$).

Within F1, the elastic bending modulus was found to differ significantly for unixed-fresh samples from the medial and the ventral regions ($p = 0.006$), for samples fixed with ethanol from the lateral and the ventral regions ($p = 0.024$) as well as from the dorsal and the ventral regions ($p = 0.030$) and for samples treated with mixed formalin solution from the dorsal and the ventral regions ($p = 0.049$). For F2 a difference of significant level in elastic bending modulus was observed for unixed-fresh samples from the lateral and the ventral regions ($p = 0.036$). When evaluating all tested specimens from both bones together, significant differences in elastic bending modulus between ethanol fixed bone from the medial and the ventral regions ($p = 0.050$) as well as from the lateral and the ventral regions ($p = 0.008$) and for unixed-fresh specimens from the medial and the lateral regions ($p = 0.033$) were determined.

### 4 Discussion

The present study observed the influence of commonly used chemical fixatives on the mechanical properties of unfixed rectangular bone specimens and to compare these data to region-dependent properties obtained from the specimens. Machined specimens were successfully tested in a four-point-bending test, enabling the examination of elastic bending modulus and ultimate bending strength in small specimen dimensions of a given geometrical shape and anatomical orientation.

As it is difficult to obtain, prepare and test fresh human tissues immediately after removal, the unfixed bone samples were temporarily shock frozen for storage. Though freezing the specimens for storage may be regarded as a gold standard due to minor effects on the mechanical properties of bones [1–3], other studies showed alterations of almost 30% in other tissues [16]. Furthermore, a possi-
The influence on the mechanical properties due to the loss of water [15] was minimized by rinsing the specimens prior to mechanical testing.

Four-point-bending was accounted to be suitable as a test method because of the constant bending moment between the loading points. Furthermore, no transverse shear stresses are present like they are in a three-point-bending test around the middle section of the specimen [17]. However, this method requires the force to be equal at both loading points, which is quite difficult to accomplish when the entire bone is tested. Nevertheless, entire bones are often used in experimental setups and they might be easier to handle. However, using entire bones, only extrinsic properties and the overall behavior of the bone can be calculated with the widely applied techniques of tensile or compressive material testing. The further deduction of intrinsic mechanical properties remains very challenging, since the geometrical shape of each bone varies inter-individually and possibly age-dependently. Therefore, a precise reassembly of entire bone material properties is quite time-consuming and due to the shape irregularities a large amount of samples is required to make reliable statistical predictions. Here, machined specimens at a pre-defined size are advantageous.

Given the large amount of different donor species, testing conditions and fixation media, the results of most studies are hard to compare. Our results are quite similar to the data of van Haaren et al. [3], who also found minor and non-significant differences in bending strength in unfixed and formaldehyde-fixed entire bone specimens from goats. However, using faunal bones to derive conclusions for the human locomotive system remains critical, since there are species- and geometry-dependent material properties of cortical bone [18]. Therefore, the most reliable data has to be derived from human bone tissues.

In summary, the possible influence of different fixatives on the mechanical properties of human cortical bone specimens was examined under standardized and reproducible testing conditions. The lack of significant differences between unfixed-fresh, ethanol- and mixed-formalin-fixed bone specimens suggests that the given fixation agents can be used in the short-term, if data close to the native situation is of interest and if the samples are sufficiently rinsed in isotonic saline prior to the material testing.

In conclusion, we see our first hypothesis disproved since no significant differences comparing the three fixation methods could be determined. On the other hand, our investigations corroborated the second hypothesis that mechanical properties bone depend on the anatomical region of the specific samples.

In further investigations the duration of fixation should be extended to examine possible long-term effects. Moreover, the influence of fixative solutions on the mechanical properties of cancellous bone might be of interest.

Author’s Statement

Conflict of interest: Authors state no conflict of interest.

Material and Methods: Informed consent: Informed consent has been obtained from all individuals included in this study. Ethical approval: The research related to human use has been approved with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee.

References


