Acta of Bioengineering and Biomechanics
Vol. 17, No. 1, 2015

Original paper
DOI: 10.5277/ABB-00067-2014-03

Influence of three different preservative techniques on the mechanical properties of the ovine cortical bone

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Purpose: Preservative treatments are necessary for disinfection and long term storage when dealing with biological tissue. Freezing is a gold standard but infectious risk can only be eliminated by using chemical fluids that may alter the mechanical properties, depending on their composition. Therefore, we experimentally evaluated the influence of freezing and of two commonly used preservative fluids (formalin and alcohol) on the intrinsic mechanical properties of ovine cortical bone samples, compared to purely fresh samples. Methods: Prismatic specimens were prepared from the sheep's metacarpal bones and were divided into four groups (fresh, fresh-frozen, formalin and alcohol). All samples underwent four-point-bending; fresh samples were tested immediately, preserved samples were tested after 14 days. Bending modulus, bending strength, yield strength and energy absorption for the elastic and plastic region were determined. Results: Significant differences were found for the plastic energy absorption for formalin (–41%) and alcohol (+37%) preservation compared to fresh samples. Formalin preservation revealed embrittlement of the cortical bone samples and alcohol preservation revealed higher ability of plastic energy absorption. Conclusions: Our results indicate that freezing has no influence on the mechanical properties of the ovine cortical bone. Preservation with chemical fluids (formalin and alcohol) showed no influence on the elastic properties but it was observed for the ability of plastic energy absorption. Therefore, these methods seem to be suitable for preservation without evident altering of the elastic mechanical properties.

Key words: *mechanical properties, chemical preservatives, cortical bone, freezing, four-point-bending, fresh bone samples*

1. Introduction

In the field of biomechanics and biomedical engineering, developing and investigating new types of implants or determining the implant–tissue interaction requires various mechanical testing as well as in vitro experiments. These experiments should be conducted in an environment that simulates realistic in vivo conditions in order to obtain meaningful results. Therefore, the use of biological and human tissue is necessary in order to mimic these conditions as closely as possible in the in vivo conditions.

Fresh samples would represent the best condition to guarantee the original behavior, but they are only of restricted usability. Furthermore, testing has to be performed immediately after harvesting, and execution of long-term tests with unpreserved samples is nearly impossible due to decomposition [9]. Therefore, bones and derived samples and specimens are often stored in a frozen condition (at minus 20 °C or below) as this gold standard procedure has been shown not to significantly affect the natural mechanical properties of bony material [4], [6], [8], [9], [12], and it provides consistent results even over long periods of time – for up to two years and in temperatures of less than –70 °C [5], [10]. But the availability of fresh-frozen bone specimens is limited, and the risk of infections persists.

Therefore, specimens are often treated with different chemical preservative fluids in order to minimize

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Received: March 24th, 2014
Accepted for publication: August 22th, 2014

the infectious risks. Subsequently, mechanical testing is performed to determine the influence of the preservative technique on the mechanical behavior. Although many studies have investigated the influence of different preservation techniques, they have rarely been carried out under standardized conditions. Thus distinct findings are still needed because the composition of the chemical fluids is often different. Furthermore, the outcomes of the studies have often been limited to only a few mechanically investigated parameters and are strongly affected by the testing protocols.

While Burkhart et al. [2] found higher axial and torsional stiffness for formalin-fixed samples than for fresh-frozen samples, Goh et al. [4] ascertained that freezing and embedding in formalin had no significant influence on the stiffness in four-point-bending and torsion but showed a decrease of energy absorption as well as the bone's embrittlement. Also Nazarian et al. [12] found no differences in the static mechanical properties for fresh-frozen and formalin-fixed specimens compared to purely fresh samples but a decrease in loss and dynamic modulus for formalin-fixed samples.

Many studies performed testing of entire bones and bone segments, a common way of bone testing. While the testing of entire bones always includes the influence of the bones' geometry, testing is often conducted with machined samples by means of compression or three- and four-point-bending in order to investigate the intrinsic material properties. But also for this testing procedure, contradictory results have been found.

McElhaney et al. [11] found a significant decrease, by 12%, in the compressive strength for rectangular, prepared bone samples treated with a fixation fluid containing alcohol and formalin, compared to fresh samples – with which Ohman et al. [14] found no significant decrease in ultimate compression strength but a decrease of Young's modulus by 24%. Under three-point-bending conditions, Kikugawa et al. [7] found an increase in bending stiffness by 30% and decrease in bending strength by 11% for formalin fixation. In contrast, Unger et al. [17] and Currey et al. [3] did not find any significant changes for bending modulus or for bending strength.

Since many studies have been performed studying different aspects, finding a general answer to the influence of the chemical fluids is difficult. Some investigations were conducted with human bones [2], [9], [14], [17], and others with faunal bones [3], [4], [7], [11], [12], [17], [18]. Moreover, the investigations were either obtained from entire bones [4], [12], [15], [18], bone segments [2], [12] or from machined samples with standardized geometrical shapes [3], [7], [9], [11], [14], [17].

Since the mechanical properties seem to be strongly dependent on the composition of the chemical fluid, we therefore investigated the influence of the chemical embalming fluids being used as the standard solution at the local Department of Anatomy. The influence of three preservation techniques (fresh-frozen and embalming with formalin or alcohol) on the mechanical properties of the ovine cortical bone were investigated under standardized, reproducible conditions in a four-point-bending test and were compared to purely fresh, harvested samples. Here, mechanical properties for the elastic and plastic behavior as well as the energy absorption were investigated in order to give a general overview about the influence of the preservation method.

2. Materials and methods

2.1. Specimen harvesting and preparation

The metacarpal bone from five, three-year-old female sheep (breed: "Schwarzkopf Fleischschaf") with a weight of 71.2 ± 5.4 kg were harvested after sacrifice for a study unrelated to ours (implantation of open-porous titanium scaffolds within the metatarsal bone with no effects on bone metabolism or mechanical properties). Both intact forelegs were taken in order to consider possible differences in the bone quality between the left and the right sides due to unrelated, previous surgical interventions on the right hind limbs of the sheep. Each bone was dissected and soft tissue (skin, tendons, periosteum) was carefully removed from the bone surface.

Immediately after harvesting, we conducted a sample preparation of the bone specimens. Rectangular rods were cut out of the substantia compacta with a diamond bandsaw (Type 28186, MBS 240/E, Proxxon GmbH, Foehren, Germany), according to a DIN standard guideline (DIN EN 843-1:2008-08) for four-point-bending testing of ceramic high-performance materials. Self-developed equipment was used to support the sample preparation and to ensure the comparability of the prepared samples' geometrical shape.

Machined bone samples were prepared with the beam axis orientated to be parallel to the longitudinal axis of the sheep's metatarsal bone (Fig. 1). Hence,

loading was performed in the samples' transversal direction. The edge lengths of the samples were 1.84 ± 0.18 mm in height and 1.81 ± 0.18 mm in width, with a length of at least 25 mm. This ratio of length to width is sufficiently large to neglect shear effects during testing. No further surface treatment was performed due to the samples' good surface quality, because of the diamond saw. However, it is known for the determination of the mechanical properties that unpolished samples exhibit approximately the same mean but twice the standard deviation of the polished samples [6].

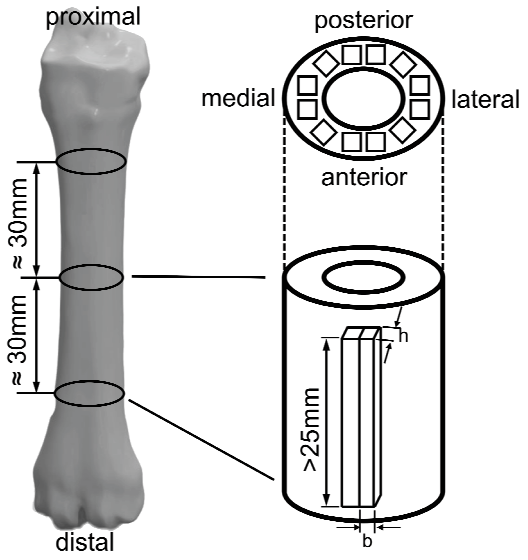

Fig. 1. Preparation scheme for the ovine bone samples. Samples were prepared approximately 30 mm proximal and distal from the mid-diaphysis. Rods with a height (*h*) and width (*b*) of 2 mm were cut out of the compacta with the orientation in radial direction. Length of the samples was parallel to the bones' vertical axis

2.2. Preservation and storage

The prepared samples were randomly assigned to four different groups (fresh, fresh-frozen, formalin and alcohol). Samples with poor geometrical shapes (containing notches, varying cross-sections along the axis, non-rectangular cross-sections) were removed prior to assigning them to one of the four groups. Care was taken to ensure that a similar number of rods with optimal geometrical shapes were placed in each group. In total, 244 samples were successfully turned into suitable test samples and stored in accordance with the specifications listed below. The fresh samples were tested directly following the preparation procedure. All other samples were stored for 14 days in 2 ml tubes (Eppendorf AG, Hamburg, Germany) according to these preservation methods:

(a) Fresh specimens (FR): The specimens were immersed in a 0.9% sodium chloride solution at room temperature in order to avoid dehydration.
(b) Fresh-frozen specimens (FF): The specimens were stored in a freezer at minus 20 °C.
(c) Formalin preservation (FO): The formalin fixative used for this study consisted of 9 parts of distilled water, 22 parts of ethanol (96%), 3 parts of glycerin, 2 parts of formalin (37%), 0.15 parts of thymol, and 0.1 parts of salicylic acid (this is the standard solution used at the local anatomical department).
(d) Alcohol preservation (AL): The alcohol fixative used for this study was an alcohol solution containing 96% ethanol.

The corresponding 2 ml tubes for the formalin and the alcohol groups were filled with preservation fluid covering the entire bone specimen and stored at room temperature for 14 days. All of the specimens, the anatomical sides, and the preservative techniques are listed in Table 1.

Table 1. Number of samples prepared for the four-point-bending testing

Animal No.	Preservative technique *	Left side	Right side
# 1	FR/FF/FO/AL	7/7/7/7	5/5/5/4
# 2	FR/FF/FO/AL	5/5/5/5	$4/4^{(+)}/4^{(++)}/4$
# 3	FR/FF/FO/AL	5/5/5/4	6/5/6/6
# 4	FR/FF/FO/AL	8/8/8/6	8/7/8/8
# 5	FR/FF/FO/AL	8/8/7/8	5/5/4/5

* FR: Fresh, FF: Fresh-frozen, FO: Formalin preservation, AL: Alcohol preservation

$^{(+)}$ Bone samples were stored at room temperature for 14 days instead of freezing (decomposed samples)

$^{(++)}$ Formalin fixed samples were additionally stored in the freezer at minus 20 °C for 14 days.

The samples of sheep #2 (only samples from the right side) of the fresh-frozen group (n = 4) were inadvertently stored for 14 days at room temperature (decomposed samples). In return, the specimens of the formalin group (n = 4) were additionally stored at minus 20 °C. These specimens were analyzed separately in order to determine the influence of the decomposition process for untreated samples compared to the results of the fresh samples from the same side. Hence, statistical analysis of the influence of the preservative treatment was only performed on the remaining 236 samples. Nevertheless, all samples were tested under four-point-bending conditions.

2.3. Preparation for mechanical testing

Prior to mechanical testing, the frozen samples were thawed at room temperature, and the fixed ones were removed from the preservation fluid. Nyman et al. [13] demonstrated the influence of dehydration on the mechanical properties. Therefore, all specimens were re-hydrated by being immersed in isotonic saline (0.9% sodium chloride, B. Braun Melsungen AG, Melsungen, Germany) at room temperature for at least one hour prior to testing, in order to avoid any influence due to dehydration. The intrinsic height h and width b of each sample were obtained using a caliper gauge.

2.4. Mechanical testing

All specimens, treated and untreated, underwent a four-point-bending test conducted in an electromechanical testing machine (Z1.0, Zwick GmbH & Co. KG, Ulm, Germany) according to ASTM standard (ASTM 6272) with a cross head speed of 1 mm/min. A four-point-bending test was considered suitable for testing brittle materials due to the area of constant bending moment and absent shear forces [1], [16]. The test setup is shown in Fig. 2.

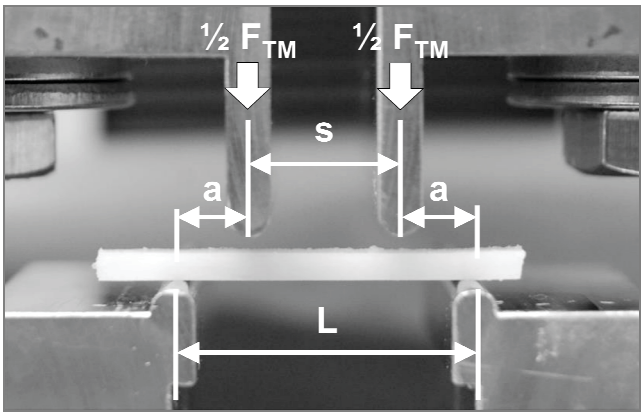

Fig. 2. Four-point-bending test setup with positioned bone sample. Position of bone, supports and loading nose prior to testing. The support span L = 20 mm, the loading span s = 10 mm, the lever arm a = 5 mm, the curvature of the load, and the support bearing exhibited a radius of 1.5 mm. Note that the applied load on each loading span is half the load of the testing machine F_{TM}

A preliminary loading without specimens was performed in order to account for the compliance of the test setup and the testing machine. This adjustment curve was applied for all testing procedures, and doing so eliminated the machine's undesired deformations from pure deformations. This was especially important for testing procedures with only small displacements and high loads (i.e., bending and compression testing).

A preload of 2 N was applied to the samples. After a dwelling of 2 s, the specimens were loaded at a crosshead speed of 1 mm/min until failure.

Values of applied load and displacement of the traverse were continually recorded during testing. Bending strength and elastic bending modulus as well as energy absorption for the elastic and the plastic regions were calculated.

2.5. Calculating the mechanical properties

As the dimensions of height and width were approximately one-tenth of the length, the rod was treated as a beam, undergoing bending deflection due to the applied load. Furthermore, the Euler–Bernoulli bending theory was used (no shear deformation). Thus, Young's modulus was calculated as follows

$$E = \frac{2}{3} \cdot \frac{\Delta F_{TM} \cdot a^3}{I_y \cdot \Delta w} \tag{1}$$

where I_y is the area moment of inertia (i.e., 1/12 bh³), a is the lever arm (5 mm), and $\Delta F_{TM}/\Delta w$ is the linear slope of the force–displacement relationship.

The ultimate stress was calculated by the following

$$\sigma_{\max} = \frac{3 \cdot F_{TM,\max} \cdot a}{b \cdot h^2} \tag{2}$$

where $F_{TM,\max}$ is the maximum applied load of the testing machine immediately upon the specimens' failure.

Furthermore, the energy absorption until the failure for each specimen was calculated as a line integral, being equivalent to the area under the load-displacement relationship.

$$W = \int_0^{w_{\max}} F(w)dw\,. \tag{3}$$

As this value strongly depends on the dimensions of the specimens, the values are referred to a correlation factor C_F, thus resulting in a standardized value. This factor took into account the geometric dimension for conversion from load–displacement to stress–strain relationships, offering normalized values for independent comparison W_{norm}.

$$W_{\text{norm}} = C_F \cdot W \tag{4}$$

where C_F is $\left(\frac{9}{8} \cdot \frac{1}{b \cdot h \cdot a}\right)$.

In order to distinguish between elastic and plastic energy absorption, the corresponding deflection point was identified by using the 0.2% strain offset [16]. Hence, two different line integrals were calculated for the elastic region corresponding to a 0.2% strain and for the plastic region up to $\sigma_{\max}$.

2.6. Statistical analysis

Data determined from the experimental testing were analyzed for all parameters using statistical software (SPSS, version 20, IBM Corp., Armonk, NY, USA). Statistical calculations were performed for the five parameters investigated with respect to the four groups (fresh, fresh-frozen, formalin, alcohol) and for the anatomical sides (right and left forelegs).

In a first step, a one-way ANOVA test was performed to determine whether the anatomical side revealed significant differences for any of the parameters investigated. As this was not the case, the data from the specimens were pooled only with respect to the four groups (fresh, fresh-frozen, formalin and alcohol). A pair-wise Mann–Whitney test was then performed in order to determine which preservative technique had a significant influence on the mechanical properties. *P*-values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Failure characteristics

Failure for all specimens occurred within the region of the loading span. Most of them failed with vertical breakage through the whole specimen, while some of the specimens showed a kind of delamination with a horizontal breakage line along the specimens' axes. In general, four different types of load–displacement relationships (not correlating with the preservation methods) occurred during testing (Fig. 3).

The first type was characterized by a steady linear increase with sudden failure. The second type also showed a steady increase, passing over into being nonlinear, but sudden failure also occurred. For the third type, the load–displacement relationship revealed a steep decrease after reaching the maximum load, without complete failure. Further bending was possible, but the applied load could not be increased any further. For the first three types, the failure load was equal to the maximum applicable force by the testing machine $F_{TM,\max}$ and was recorded during testing. The fourth type also showed a decrease (negative value for the first derivative) in the load–displacement relationship, but thereafter, the loading increased further. In this case, the failure load was defined for the first indication of subsidence (negative slope) within the curves and was not the maximum applicable force by the testing machine. Although four different failure characteristics were identified, they did not correlate with the four preservation methods. Failure for most of the samples of each group occurred by types 1 and 2.

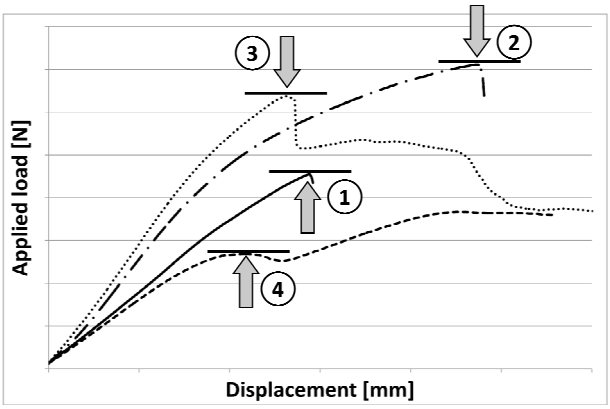

Fig. 3. Failure characteristics of cortical bone samples. Schematic illustration of four different types of failure and the determination of the failure load (horizontal lines and grey arrows), distinguished with encircled numbers

3.2. Influence of decomposition process

The bone samples of the fresh-frozen group from the right side of animal # 2 (n = 4, please refer to Table 1) were stored for 14 days at room temperature without preservative method and putrid smell of the decomposition processes were clearly noticeable. Nevertheless, compared to the results of fresh samples from the same side (right), the mean value of the bending modulus did not change due to the decomposition, but a higher standard deviation (SD) was found (Fig. 4). The bending strength and the yield strength decreased by 6.6% and 1.1%, respectively (not statistically significant). A higher SD was also found for the bending strength, and a lower SD was found for the yield strength for the decomposed specimens than for the fresh samples. Specific energy absorption de-

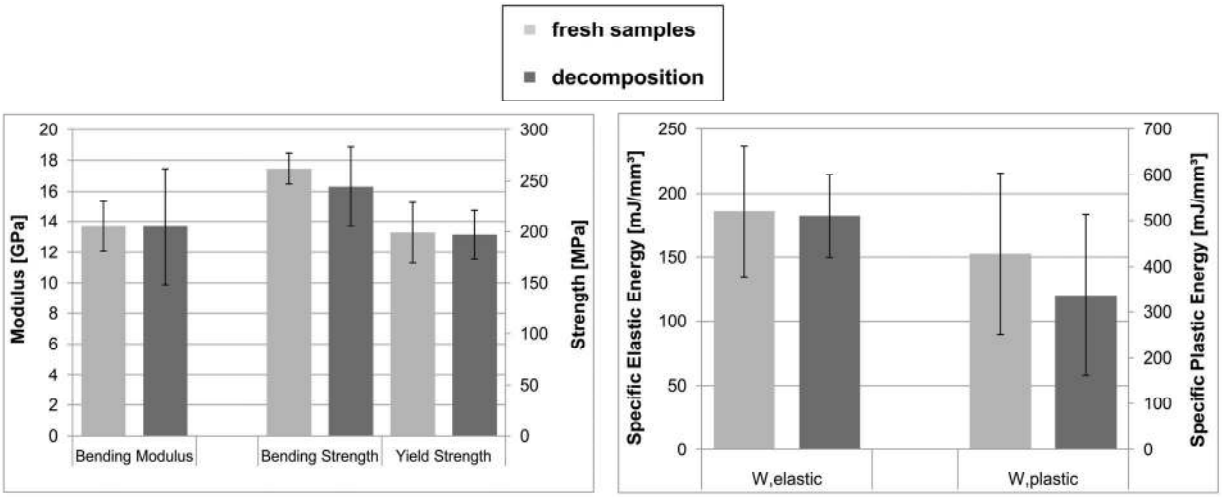

Fig. 4. Influence of the decomposition process on the mechanical properties. The results of the testing for fresh samples (light grey) and the samples stored at room temperature for 14 days without preservative fluid (dark grey). Left: Mechanical properties (bending modulus, bending strength and yield strength). Right: Fracture properties (specific elastic and plastic energy absorption until failure)

creased by 1.9% and by 21.1%, respectively, for the elastic and the plastic regions (also not statistically significant due to the small number of samples). A lower SD was found for the elastic energy, and a similar SD was found for the plastic energy absorption.

3.3. Statistical evaluation of the anatomical side

Statistical analysis confirmed that the anatomical side (right or left foreleg) was not significantly different for any of the parameters investigated (One-way ANOVA: $\gg 0.05$). Therefore, results were pooled and analyzed only with respect to the four different treatment methods (fresh (n = 62), fresh-frozen (n = 57), formalin (n = 57) and alcohol (n = 60)).

3.4. Influence of the preservation technique on the mechanical properties

Results for the five mechanical properties investigated are summarized as boxplots in Fig. 5. For all parameters under investigation, the mean and median values are close to each other, indicating good symmetries in the distribution of the results. Significant differences are indicated with square brackets between the corresponding results.

Mechanical testing revealed 17.8 ± 3.5 GPa and 256.0 ± 33.1 MPa, respectively, for the bending modulus and the bending strength of the fresh cortical bone samples. No significant difference was found due to the preservation method for those two mechanical parameters. Nevertheless, the formalin preservation showed slightly higher values than the other preservative techniques, and a higher grade of inhomogeneous material property distribution (e.g., a 9.7% increase in the bending modulus compared to the fresh samples).

A similar effect occurred for the specific elastic energy absorption. Here, again slightly higher values (not significant) than the fresh samples were found for the formalin preservation (+3.3%) and slightly lower values for the alcohol preservation (–8.6%), with a more homogeneous distribution.

By formalin preservation, significantly lower values were found in the specific plastic energy absorption compared to the fresh (–41.0%), the fresh-frozen (–51.1%) and the alcohol samples (–56.9%), each with $p < 0.001$. Furthermore, 22.8% of the formalin-preserved samples showed no ability for plastic energy absorption (i.e., 0 mJ/mm^3). In contrast, preserving the samples with alcohol revealed a significant increase (+37.1%, $p = 0.024$) and a less homogeneous material property distribution compared to the fresh samples.

A significant difference in yield strength was found only between alcohol preservation and formalin preservation (–9.5%, $p = 0.006$). Furthermore, alcohol preservation showed slightly lower and formalin fixation slightly higher values than the fresh or the

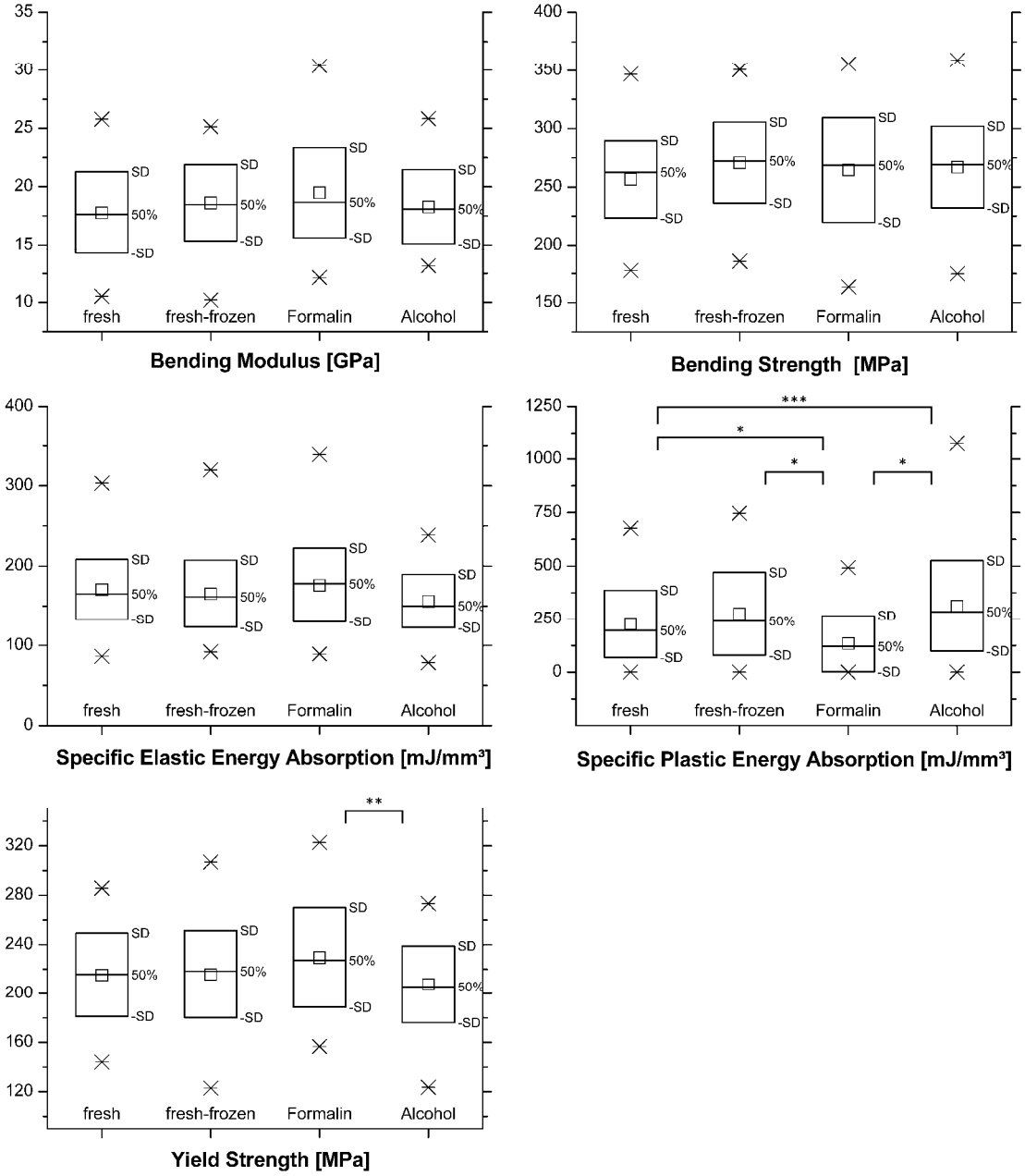

Fig. 5. Influence of the preservation on the five mechanical properties investigated.
The results of the calculated mechanical properties are shown as boxplots for the four different specimen treatments (fresh, fresh-frozen, formalin and alcohol). The mid-line of the box represents the median value, the hollow square represents the mean value, and the upper and lower box lines represent the area of the standard deviation (SD). The cross marks stand for the minimum and maximum values. Square brackets indicate significances with the *p*-values given below for the corresponding data (marked with asterix) * $p < 0.001$, ** $p = 0.006$, *** $p = 0.024$

fresh-frozen samples. But neither observation was significant.

4. Discussion

In the present study, we verified that freezing did not have an influence on any of the parameters investigated. Furthermore, we found that the utilized chemical fluids did not have a significant influence on the elastic mechanical properties but on the plastic energy absorption and on the yield strength. Here, the formalin-preserved samples showed a significant embrittlement due to the loss in the ability of plastic energy absorption and alcohol preservation revealed higher plastic energy absorption.

For investigating the mechanical properties, four-point-bending was considered a suitable test method due to the region of pure bending between the two loading points without the presence of transverse shear stresses. In contrast, for the three-point-bending test, high shear stresses near the middle section of the specimens occurred [1], [16]. Nevertheless, the force at both loading points must absolutely be equal, which requirement is difficult to achieve in entire bone testing. Because prepared bone samples with defined geometrical shapes were used instead of entire bones, the intrinsic material properties were calculated.

Dealing with fresh samples is often restricted due to the limited availability, the infectious risk and the risk of alteration in the mechanical properties due to the decomposition process without preservative treatment. Linde and Sorensen [9] observed that the compressive stiffness of the trabecular bone decreased by 10% within the first 24 hours post mortem when stored only in physiological saline at room temperature. In contrast, our results from the testing of the decomposed cortical bone samples that were stored for 14 days at room temperature without any preservative method revealed no difference in the elastic properties under investigation. A small decrease was only found for the plastic energy absorption. Nevertheless, preservative methods should be used when samples need to be stored for a certain time.

Freezing is the gold standard for preserving bone samples over a certain time without influencing the mechanical properties. Since studies are often performed with entire bone testing [4], [12], [18], we additionally investigated the influence of freezing on the intrinsic mechanical properties of cortical bone samples with regular shapes.

The results from Kaye et al. [6] and Kuninori et al. [8] showed no influence due to the freezing process but the studies were performed either with specific devices or with a focus of only one mechanical parameter (fracture toughness). Furthermore, the freezing temperature and storage duration from the work of Kuninori et al. [8] were slightly different (minus 30 °C for 30 days). Nonetheless, their findings agreed with our results that freezing for two weeks, thawing and rehydration in saline solution did not affect the investigated mechanical properties of the cortical bone. None of our parameters showed any significant difference between the fresh and the fresh-frozen samples.

Comparing the influence of the preservative fluid with other studies is difficult due to the absence of standardized testing procedures for bone tissue and preservation techniques. In addition to the differences in testing setups (for entire bones or machined samples), the following are various factors influencing the results and making it hard to compare with each other: the testing method (compression, tension, torsion, three- or four-point-bending), species (human or faunal), chemical composition of the preservative fluid (4% or 10% formalin, neutral buffered formalin with or without additives, alcohol), storing time (3 h, up to two years), preconditions (e.g., initial fresh-frozen condition and then additional preservative treatment), and whether the samples were rehydrated in a sodium solution after preservative treatment. Moreover, published data revealed controversial findings whether the use of the chemical fluid has got an influence on the mechanical properties or not [2], [4], [12], [18].

Burkhart et al. [2] found a significant increase in axial and in torsional stiffness by 14.1% and by 14.3%, respectively, of formalin preservation (4% for six weeks) compared to non-treated human femoral bones.

In contrast, the results of Goh et al. [4] revealed no significant difference in bending and torsional stiffness for feline bones due to formalin fixation (4% for 3 and 21 days). But they found a significant decrease for energy absorption, by 40% in torsion and by 50% in bending for the formalin-treated samples. Whether the differences occurred for the elastic or the plastic energy absorption was not investigated.

Nazarian et al. [12] also investigated the influence of 10% formalin preservation on the dynamic properties of murine femora and vertebral bodies under bending and compression. They concluded that two weeks of embedding does not have an influence on stiffness, modulus of elasticity, yield stress and ultimate stress, but it resulted in a decrease of loss and in dynamic moduli.

Van Haaren et al. [18] found no statistically significant differences for bending and for torsion in stiffness and strength for none of their observed time points up to one year due to storage in formalin (10%).

Although controversial statements can be found for the use of chemical fluids the use of 4% and 10% formalin for different time periods seems in general not to have a significant influence, in the case of entire bone testing. However, the mechanical properties investigated, i.e., bone stiffness and energy absorption, depend on the geometrical dimensions of the specimens. Since the inner dimensions of the bone geometry are hard to determine (e.g., CT or μ-CT data) the results of the testing reveal the mechanical behavior of the bone structure and may be influenced by the geometry, i.e., the variation of the moment of inertia [12].

Therefore, we investigated mechanically prepared bone samples with a regular shape in order to determine the intrinsic material properties of the cortical bone. Results from our mechanical testing showed no significant differences in the elastic material properties (bending modulus and bending strength) when preserving samples with formalin or alcohol for a relatively short time (14 days). Since the composition of the chemical fluids used at our department was different from commercially available products, a direct comparison is difficult.

Moreover, we found significant differences for the plastic energy absorption for the treatment with chemical fluids. Embrittlement (less plastic energy absorption) of the samples was observed due to the treatment with formalin, and higher energy absorption was observed for alcohol fixation.

Kuninori et al. [8] found a significant decrease in the fracture toughness of up to 18% for machined bovine cortical bone samples stored for 30 days with formalin and neutral, buffered formalin.

Unger et al. [17] also investigated different preservation methods via three-point-bending and found some significant differences due to some of their fixation methods. Here, the formalin-fixed specimens showed a significant decrease in ultimate strain and plastic energy absorption compared to the fresh-frozen samples after a period of six months. Furthermore, alcohol fixation showed significantly higher plastic energy absorption.

Kikugawa et al. [7] investigated the effects of formalin on the properties of cortical bovine bone samples in a three-point-bending test. Their results showed a significant increase of the bending stiffness, by 30%, and a decrease of the bending strength, by 11%, after storage in formalin for 140 days. Moreover, fracture toughness decreased by 20% immediately after fixation.

Ohman et al. [14] performed compressive testing on human femoral bones. Samples were preserved with 4% formalin for three time periods: for 24 hours, for four weeks, and for eight weeks. While no significant difference regarding ultimate strength occurred for any of the time points investigated, Young's modulus decreased significantly only for the longest investigated preservation period of eight weeks.

Nevertheless it has to be considered that in our study the preservation time was only 14 days. The influence due to the chemical preservation fluid on the mechanical properties could increase for a preservation time of more than two weeks.

It is very difficult to obtain, to prepare, and to test or to treat pure fresh bone samples. Although the differences between different preservation techniques were investigated, most specimens are stored in a frozen condition first [14], [17]. Although the mechanical properties are not affected by freezing, a subsequent treatment with chemical fluids could alter the outcome as embalming with chemical fluids after pre-frozen conditions might influence the mechanical properties. Concerning this fact, the influence of the preservation fluids used on the mechanical properties cannot be objectively rated in the same way as results obtained from absolutely fresh bone.

5. Conclusion

Our present results revealed that freezing has no significant influence on the mechanical properties of cortical bone. Thus, fresh-frozen samples can be unhesitatingly used for sample storage but with remaining infectious risk. Moreover, preservation in chemical fluids like formalin and alcohol for 14 days did not show a significant influence for the elastic parameters. An influence due to formalin preservation was only found in a significantly reduced ability of plastic energy absorption. Hence, preservation in formalin resulted in the embrittlement of bone. In contrast, the alcohol preserved samples showed a higher ability of plastic energy absorption. This circumstance was also supported by the significant difference in yield stress between formalin and alcohol preservation. Moreover, the cortical bone revealed no signs of decay for the elastic material properties after a period of 14 days without any preservative fluid or freezing.

References

[1] ATHANASIOU K.A., ZHU C.F., LANCTOT D.R., AGRAWAL C.M., WANG X., *Fundamentals of biomechanics in tissue engineering of bone*, Tissue Engineering, 2000, 6(4), 361–381.

[2] BURKHART K.J., NOWAK T.E., BLUM J., KUHN S., WELKER M., STERNSTEIN W. et al., *Influence of formalin fixation on the biomechanical properties of human diaphyseal bone*, Biomed. Tech. (Berl.), 2010, 55(6), 361–365.

[3] CURREY J.D., BREAR K., ZIOUPOS P., REILLY G.C., *Effect of formaldehyde fixation on some mechanical properties of bovine bone*, Biomaterials, 1995, 16(16), 1267–1271.

[4] GOH J.C., ANG E.J., BOSE K., *Effect of preservation medium on the mechanical properties of cat bones*, Acta Orthop. Scand., 1989, 60(4), 465–467.

[5] HAMER A.J., STRACHAN J.R., BLACK M.M., IBBOTSON C.J., STOCKLEY I., ELSON R.A., *Biochemical properties of cortical allograft bone using a new method of bone strength measurement. A comparison of fresh, fresh-frozen and irradiated bone*, J. Bone Joint Surg. Br., 1996, 78(3), 363–368.

[6] KAYE B., RANDALL C., WALSH D., HANSMA P., *The effects of freezing on the mechanical properties of bone*, Open Bone J., 2012, 4(1), 14–19.

[7] KIKUGAWA H., ASAKA T., *Effect of long-term formalin preservation on bending properties and fracture toughness of bovine compact bone*, Materials Transactions, 2004, 45(10), 3060–3064.

[8] KUNINORI M., KIKUGAWA H., ASAKA T., KASUYA H., *Effect of Different Preservative Methods on Fracture Behavior of Bovine Cortical Bone*, Materials Transactions, 2009, 50(2), 305–312.

[9] LINDE F., SORENSEN H.C., *The effect of different storage methods on the mechanical properties of trabecular bone*, J. Biomech., 1993, 26(10), 1249–1252.

[10] MATTER H.P., GARREL T.V., BILDERBEEK U., MITTELMEIER W., *Biomechanical examinations of cancellous bone concerning the influence of duration and temperature of cryopreservation*, J. Biomed. Mater. Res., 2001, 55(1), 40–44.

[11] MCELHANEY J., FOGLE J., BYARS E., WEAVER G., *Effect of embalming on the mechanical properties of beef bone*, J. Appl. Physiol., 1964, 19, 1234–1236.

[12] NAZARIAN A., HERMANNSSON B.J., MULLER J., ZURAKOWSKI D., SNYDER B.D., *Effects of tissue preservation on murine bone mechanical properties*, J. Biomech., 2009, 42(1), 82–86.

[13] NYMAN J.S., ROY A., SHEN X., ACUNA R.L., TYLER J.H., WANG X., *The influence of water removal on the strength and toughness of cortical bone*, J. Biomech, 2006, 39(5), 931–938.

[14] OHMAN C., DALL'ARA E., BALEANI M., VAN SINT J.S., VICECONTI M., *The effects of embalming using a 4% formalin solution on the compressive mechanical properties of human cortical bone*, Clin. Biomech. (Bristol, Avon.), 2008, 23(10), 1294–1298.

[15] TOPP T., MULLER T., HUSS S., KANN P.H., WEIHE E., RUCHHOLTZ S., ZETTL R.P., *Embalmed and fresh frozen human bones in orthopedic cadaveric studies: which bone is authentic and feasible?* Acta Orthop., 2012, 83(5), 543–547.

[16] TURNER C.H., BURR D.B., *Basic Biomechanical Measurements of Bone – A Tutorial*, Bone, 1993, 14(4), 595–608.

[17] UNGER S., BLAUTH M., SCHMOELZ W., *Effects of three different preservation methods on the mechanical properties of human and bovine cortical bone*, Bone, 2010, 47(6), 1048–1053.

[18] VAN HAAREN E.H., VAN DER ZWAARD B.C., VAN DER VEEN A.J., HEYLIGERS I.C., WUISMAN P.I., SMIT T.H., *Effect of long-term preservation on the mechanical properties of cortical bone in goats*, Acta Orthop., 2008, 79(5), 708–716.