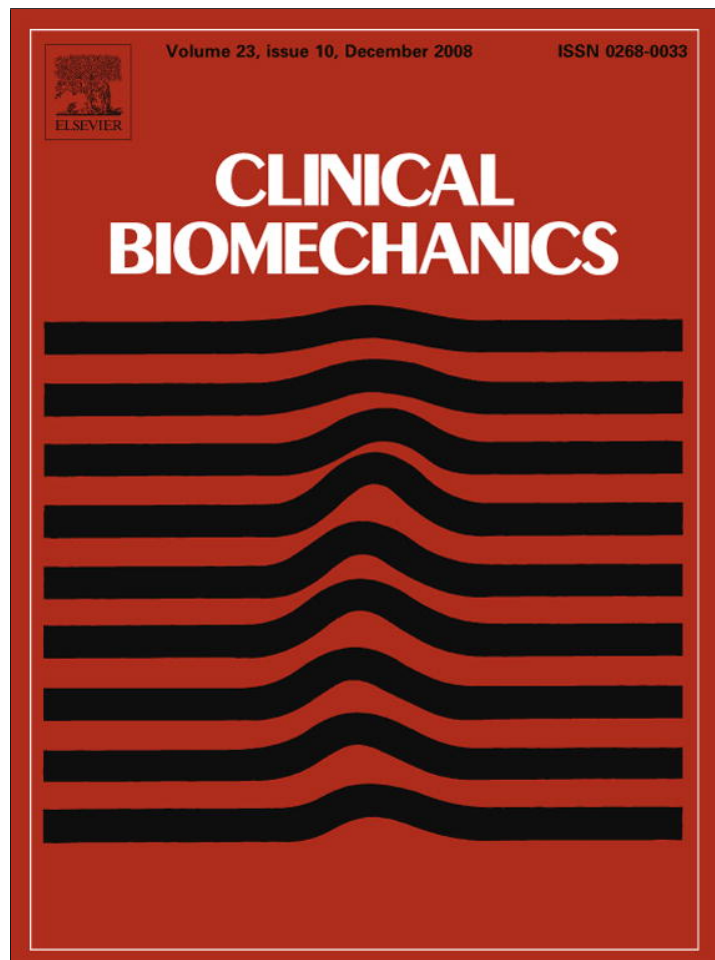


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The effects of embalming using a 4% formalin solution on the compressive mechanical properties of human cortical bone

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Abstract

Background. The use of formalin fixed bone tissue is often avoided because of its assumed influence on the mechanical properties of bone. Fixed bone tissue would minimise biological risks and eliminate preservation issues for long duration experimental tests. This study aimed to determine the short- and long-term effects of embalming, using a solution with 4% formalin concentration, on the mechanical properties of human cortical bone.

Methods. Three-millimetre cylindrical specimens of human cortical bone were extracted from two femoral diaphyses and divided in four groups. The first group was used as control, the remaining three groups were left in the embalming solution for 48 h, 4 week, and 8 week, respectively. Compressive mechanical properties, hardness and ash density were assessed. The last was used to check the homogeneity among the four groups.

Findings. No significant differences were found among the four groups in yield stress, ultimate stress and hardness. The specimens stored for 8 week in the embalming solution had significant lower Young's modulus (−24%), higher yield strain (+20%) and ultimate strain (+53%) compared to the other groups.

Interpretation. On a short-term perspective, embalming did not affect the compressive mechanical properties, nor hardness of human cortical bone, whereas a long-term preservation (8 week) did significantly affect Young's modulus, yield strain and ultimate strain in compression. Preserving bone segments for up to 4 week in an embalming solution with low formalin concentration seems to be an interesting alternative when collecting and/or managing fresh or fresh-frozen bone segments for biomechanical experiments is not possible.

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1. Introduction

The development of any new orthopaedic prosthesis requires pre-clinical validation according to international regulations. Some of the requirements established for implantable devices or some features of a specific prosthesis design can be verified only by means of experimental tests simulating *in vivo* working conditions.

Human bone must be used to mimic *in vivo* conditions as close as possible. Although fresh tissue would represent the best condition to guarantee the original viscoelastic behaviour of bone (Linde and Sorensen, 1993), management and mechanical testing of bone specimens may require some type of preservation of the bone tissue. In general, freezing or chemical fixation are being used. Chemical fixation is normally done using H₂CO based fluids, hereinafter referred to as formalin. Freezing has the advantages of not significantly altering the mechanical properties (Evans, 1973; Goh et al., 1989; Linde and Sorensen, 1993) but since it does not have an antimicrobial or embalming effect,

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biological risks remain and long duration tests at room temperature are not possible as non-fixed bone tissue would deteriorate. Formalin fixation conserves the tissue in a way that permits long duration tests and it also has an antimicrobial effect, minimising biological risks.

In the literature, the use of formalin solutions ranging in concentration from 4% to 10% can be found (Blackburn et al., 1992; Currey et al., 1995; Feipel and Rooze, 1999; Goh et al., 1989; Sedlin and Hirsch, 1966; Van Sint Jan and Rooze, 1992; Weaver, 1966; Wilke et al., 1996). Even though this technique is often used to fix bone tissue, surprisingly there are few studies concerning its effect on the mechanical properties. Currey et al. (1995) found that fixing bovine bone in a 10% formalin solution for 3 h slightly but significantly increased the bending Young's modulus (+2%) and largely decreased the impact energy (−46%). Goh et al. (1989) performed torsion tests on cat humeri and four-point bending tests on cat femora. Specimens were stored in a 10% formalin solution for 3 or 21 days. It was found that the formalin preservation did not alter the ultimate load and stiffness, but largely reduced the energy absorption capacity. Furthermore, no significant differences were found between the specimens fixed for 3 and 21 days. Sedlin and Hirsch (1966) tested cortical specimens from a human femur in tension. The specimens were elastically tested, then placed in a 10% formalin solution for 3 weeks and thereafter retested. The Young's modulus was determined and no significant difference was found between the two testing occasions. On the contrary, Evans (1973) found that embalming significantly increased the tensile strength, ultimate strain and elastic modulus, and Rockwell hardness of cortical bone from human tibiae, whereas the single shearing strength was preserved. Conversely, McElhaney et al. (1964) compared fresh bovine cortical bone specimens with specimens conserved for 15 h in four different embalming solutions with different formalin concentrations (2–28%). Both tensile and compressive tests were performed and it was found that both the tensile and compressive ultimate strength and strain, and elastic modulus decreased using all four embalming solutions. However, only the decrease in compressive strength was significant. Furthermore, Popperl et al. (1999) investigated the effect of a 4% formalin solution on the ultrasound properties of human calcanei. No significant effect on the stiffness index (SI) was found after 3 weeks in solution, whereas after 6 weeks and 6 months the SI had decreased significantly.

Hence some studies have been done to determine mainly the effects of 10% formalin fixation on the mechanical properties of bone tissue. However, to the authors' knowledge no study has been done to investigate the short- and long-term effect of a low concentration formalin solution on the mechanical properties of human bone.

The aim of the present study is to determine the short- and long-term effect of a solution with a low formalin concentration (4% i.e. the minimum value reported in the liter-

ature) on the compressive mechanical properties of human cortical bone.

2. Methods

2.1. Samples

Two human femurs were obtained from the International Institute for the Advancement of Medicine (IIAM, Jessup, PA, USA). The femurs were retrieved from two males with an age of 73 and 82 years, respectively. The femurs had been stored frozen until shipping, were received frozen and stored at between −19 °C and −21 °C until they were removed and each diaphysis was cut in eight 20 mm slices.

2.2. Embalming

The fluid used in this study for the embalming process was the modified Dankmeyer's method, a 4% formalin embalming solution (Van Sint Jan and Rooze, 1992). The slices of the two diaphyses were divided in a stratified way in four groups to reduce non-random effects, each group containing two slices from each femur. Group 1 was used as control, hence no embalming was done. These slices were left in Ringer's solution at room temperature for 24 h before specimen extraction and testing. The slices of Group 2, Group 3 and Group 4 were immersed in the embalming solution for 48 h, 4 weeks and 8 weeks, respectively. The slices were then removed and maintained in Ringer's solution at room temperature for 24 h before specimen extraction and testing.

2.3. Specimen extraction

Cylindrical specimens with a diameter of 3 mm and a height of 20 mm were extracted from the slices by means of a holed diamond-coated milling cutter. Sixteen specimens were extracted for each group (four from each slice).

2.4. Mechanical testing

The cylindrical specimen was fixed directly onto the testing machine (Mini bionix 858, MTS Systems Corp., Minneapolis, MN, USA) using an acrylic resin to ensure the alignment of the specimen with the machine axis. Care was taken not to let the acrylic resin rise up the sides of the specimen, excessive resin was removed with a scalpel. The free length was chosen to be four times the diameter (nominal 12 mm), to allow a direct measurement of strain on the specimen. Before testing, the specimen was kept an additional hour at room temperature in Ringer's solution to ensure that the specimen was hydrated. Strain measurements were done with an extensometer (gauge length of 4.6 mm, Mod. 634.31F-24, MTS Systems Corp., Minneapolis, MN, USA) attached with rubber bands to the central part of the specimen. This technique allows a more accu-

rate measurement of the tissue strain since its measurement is unaffected by the end effects. The specimen was compressively loaded to failure in displacement control. The cross-head displacement was set to 1.2 mm/s. During the test, load vs. displacement of the extensometer was registered. Stress was defined as the force divided by the average cross-section area and strain as the extensometer displacement divided by the initial gauge length of the extensometer. The Young's modulus was identified as the slope of the linear part of the calculated stress-strain curve (Keller, 1994). Yield stress and yield strain were defined with the 0.2% strain offset method (Bayraktar et al., 2004; Kotha and Guzelsu, 2003), ultimate stress as the maximum stress prior to failure (Keller, 1994) and ultimate strain as the strain corresponding to this stress.

2.5. Ashing

After testing, the cement endcaps were removed and the specimen was reduced to ash by burning the bone tissue in a muffle furnace at 650 °C for 24 h. The muffle furnace was then turned off and the specimens were left inside for additionally 24 h to reach room temperature and thereafter the ash weight was measured (Ohman et al., 2007). The ash density of the specimen was defined as the ash weight divided by the specimen volume (Kaneko et al., 2004; Les et al., 2002; Lespessailles et al., 1998). The volume of the bone specimen was determined from the diameter and total height measured before the compressive test. Ash density was believed not to change since it has been found that bone mineral density is not affected by formalin fixation (Boskey et al., 1982; Edmondston et al., 1994). Furthermore, mechanical properties are strongly correlated with tissue density (Keller, 1994; Lotz et al., 1991; Schaffler and Burr, 1988; Snyder and Schneider, 1991). Therefore, ash density was used to verify the homogeneity of the four groups.

2.6. Hardness

Hardness tests were done to assess the bone tissue quality. Each bone slice was ground using increasingly fine sandpaper number up to 2000 grit. Then the surface was polished by means of a napped cloth impregnated with diamond pastes, initiating with a grain size of 6 µm and terminating with 1 µm. All polishing was done under constant water irrigation. The hardness measurements were obtained performing micro-indentations on wet tissue by means of a Vickers diamond micro-indenter (Leica VMHT). A 100 gf load was applied to the bone specimen for 15 s. Thirty indentations were performed on the transverse surface of each bone slice (always leaving a distance of about 0.5 mm from the border and a relative distance of about 1 mm between each indentation). Hence, 120 hardness measurements were obtained from each of the four groups. As bone is an anisotropic material, the indentation diagonals may have different lengths. All indenta-

tions where one diagonal was >15% longer than the other were excluded (Hodgskinson et al., 1989).

2.7. Statistical analysis

To assure homogeneity of the four groups, the Chauvenet criterion was applied to exclude outliers in ash density. It was applied to all specimens together.

As a consequence of the low sample size of each group, a normal distribution could not be verified (Shapiro Wilks test, $P < 0.05$ for some of the parameters). Thus, a non-parametric analysis of variance (Kruskal–Wallis) and a nonparametric multiple comparison test were used for statistical analyses.

3. Results

All results from the tests of the four groups are summarised in Table 1. No significant difference in ash density was found among the four groups (Kruskal–Wallis $P = 0.84$). A significant difference in Young's modulus was found between Group 1 and 4 ($P = 0.002$) and Group 2 and 4 ($P = 0.004$). A significant difference in Yield strain was also found between group 1 and 4 ($P = 0.010$) and Group 2 and 4 ($P = 0.033$). Furthermore, a significant difference in ultimate strain was found between Group 1 and 4 ($P = 0.001$), Group 2 and 4 ($P = 0.008$) and Group 3 and 4 ($P = 0.002$). Moreover no significant differences in yield stress, ultimate stress and hardness were found among the four groups (Kruskal–Wallis $P = 0.67$, $P = 0.98$ and $P = 0.96$, respectively).

4. Discussion

The aim of this study was to determine the short- and long-term effect of a low formalin concentration embalming solution on the mechanical properties of human cortical bone. In this study, 64 cylindrical specimens of cortical bone were extracted from two human diaphyses. Young's modulus, yield stress, ultimate stress, ash density and hardness of non-embalmed specimens were compared to specimens embalmed during three different periods of time: 48 h, 4 weeks and 8 weeks, respectively. As expected, no significant difference was found in ash density among the groups.

A significant difference was found in Young's modulus, yield strain and ultimate stress among some of the groups. Group 4 had a significant lower Young's modulus and higher yield and ultimate strain than the control group. After 8 weeks in embalming solution the Young's modulus had decreased with 24% in comparison with the control value. Furthermore, the yield strain had increased with 20% and the ultimate strain with 53% in comparison with the control values. Conversely, no significant differences were found in yield stress, ultimate stress and hardness among the four groups.

Comparing the results from this study with those found in the literature is not easy. First of all there have been

Table 1
The results from all tests performed on human cortical bone of the four groups

	Ash (mg/mm ³)	Young's modulus (GPa)	Yield stress (MPa)	Yield strain (strain)	Ultimate stress (MPa)	Ultimate strain (strain)	Hardness (HV)
Group 1 (control) (n = 16)	1.11 (SD 0.08) Range 0.98–1.21	16.8 (SD 3.7) ^b	152.4 (SD 25.4) ^b	0.010 (SD 0.001) ^b	162.2 (SD 30.7)	0.015 (SD 0.004) ^b	44.3 (SD 4.6)
Group 2 (48 h) (n = 14) ^a	1.11 (SD 0.08) Range 1.00–1.22	16.6 (SD 3.4) ^b	150.2 (SD 23.3) ^b	0.011 (SD 0.002) ^b	163.3 (SD 23.6)	0.017 (SD 0.004) ^b	43.6 (SD 3.9)
Group 3 (4 week) (n = 16)	1.15 (SD 0.07) Range 0.97–1.22	15.2 (SD 3.8) ^b	144.0 (SD 27.5) ^b	0.011 (SD 0.003) ^b	162.8 (SD 25.1)	0.016 (SD 0.004) ^b	44.0 (SD 3.9)
Group 4 (8 week) (n = 14) ^a	1.11 (SD 0.06) Range 1.02–1.21	12.7 (SD 2.4) ^b	143.2 (SD 17.7) ^b	0.012 (SD 0.001) ^b	157.1 (SD 15.2)	0.023 (SD 0.007) ^b	45.1 (SD 5.9)

^a In one case (Group 4) the ash density was impossible to measure because the specimen crumbled during testing and it was not possible to ensure that all pieces were found. Therefore, this specimen was discharged. Three other specimens (two of Group 2, and one of Group 4) were excluded applying the Chauvenet's criterion to the ash density values.

^b In six cases (one of Group 1, one of Group 2, three of Group 3 and one of Group 4) Young's modulus, yield stress, yield strain and ultimate strain were not calculated since some noise in the strain measurements did not permit their accurate determination.

done few studies regarding the effect of embalming on the mechanical properties of bone and furthermore different testing techniques, specimen sizes and embalming solutions have been used. Despite these differences, this study is in line with the work of Sedlin and Hirsch (1966). In both that study and the present one, it was found that 3–4 weeks storage in an embalming solution does not significantly change the Young's modulus of human femoral bone. This study is also in agreement with the work of Goh et al. (1989). The results from both studies showed no significant differences in mechanical properties of bone tissue after storage in an embalming solution, neither after 2–3 days nor after 3–4 weeks. Furthermore, Blackburn et al. (1992) has shown that short-term (24 h) formalin fixation does not significantly affect the hardness of bovine cancellous or cortical bone, which is in agreement with the present study. Conversely, this study is in disagreement with the work of Evans (1973). This disagreement could be explained if different embalming solutions were used. In this study, the modified Dankmeyer's embalming method was applied, whereas in the work of Evans (1973) the type of embalming solution and the time in the solution were not specified. Furthermore, different testing techniques (tensile versus compression) and different bone segments (tibiae versus femora) were used. Furthermore, the present study is both in agreement and disagreement with another work (McElhaney et al., 1964). The different outcomes of the effect of embalming on the compressive strength might be explained by the fact that bones from different species (bovine versus human) and different specimen geometries (cylinders versus parallelepipeds) were used.

Only one study, known to the authors, has tested the effect of an embalming solution on bone tissue after more than one month (Popperl et al., 1999). Even though that study investigated the ultrasound properties of the calcaneal bone, it is still in line with this study, both studies found that storage in a 4% formalin solution caused a

decrease in bone tissue stiffness. It has been shown that formalin fixation alters the collagenous matrix of bone (Horan and An, 2006) and this might with time also have an effect on the deformability of bone tissue. Conversely, formalin fixation does not alter the mineral content (Boskey et al., 1982; Edmondston et al., 1994), which is supposed to play an important role for compressive strength of cortical bone. However, this might not be true when the tissue is working under other stress conditions (e.g. under tensile stress). Therefore, the negligible effect found at 8 weeks on the compressive yield and ultimate stress cannot be extended to different loading conditions.

The limitations of this study are the use of only one bone segment and the low sample number, i.e. all the specimens were extracted from two femurs. These limitations might have hidden small differences between the four groups.

5. Conclusions

In conclusion, low concentration formalin embalming solutions seem in the short-term not to have an effect on the compressive elastic, yield and ultimate properties and neither on the hardness of human cortical bone, whereas a long-term storage significantly decreases the Young's modulus and increased yield and ultimate strain without affecting the other mechanical properties. Therefore, whenever possible human bone tissue should be tested non-fixed, otherwise a short-term fixation in a low concentration formalin embalming solution can be considered to preserve the compressive elastic modulus and strength of bone tissue.

Conflict of interest statement

The authors declare that they do not have any financial or personal relationship with other people or organizations that could have inappropriately influenced this study.

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