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The effects of freeze drying and solvent dehydration on the bending strength and calcium content of cortical bone

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Objective: The aim of this study was to investigate the effects of the freeze drying and solvent dehydration allograft preservation methods on the mechanical properties of the graft material.

Methods: Bone blocks obtained from bovine humeri were divided into three groups. Samples in the first group were freeze dried, samples in the second group were dehydrated by solvents and samples in the third group were used as controls. All samples underwent three point bending test to investigate bending strength and were evaluated for calcium content.

Results: Bending strength of both the freeze-dried and solvent-dehydrated samples were found to have decreased significantly compared to that of the control group. The calcium content of the solvent dehydrated group was also significantly lower than that of the control group.

Conclusion: Our results suggested that both preservation techniques, used to dehydrate bone, have negative effects on the bending strength of bone as they decrease the mechanical properties of the graft material.

Key words: Bone grafts; freeze drying; mechanical strength; solvent dehydration.

Bone grafts, including vascularized or non-vascularized autogenous bone grafts, allogenic graft materials and alloplastic graft materials are often used for the reconstruction of bone defects in the maxillofacial region.[1]

Immediately upon removal from the body, the bone fragment loses cell viability, due to the lack of blood supply.[2] Mechanical properties of the bone begin to decrease, as well, because of the migration of minerals from the tissue to the surrounding environment, even if the viability of the cells is maintained in vitro. In addition, the technique used for cutting the bone may trigger a remodeling process in the bone, which will result in a decrease in its mechanical properties.[2,3] To prevent this, the bone tissue must be preserved in a state of homeostasis.

Even though it is possible to increase the shelf life of allogenic bone graft material, the method used for the preservation of the transplant should not affect the mechanical properties and the potential of integration with the host.[1,2]

In this study, we investigated the effects of two commonly used preservation methods, freeze drying and solvent dehydration, on the mechanical properties and calcium content of the bone graft.

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Materials and methods

Seventy-two bone blocks of 50x7x5 mm of size were prepared from 14 humeri of male bovines, aging 1 to 2 years and weighing 250 to 400 kg. Bone blocks with a rectangular cross-section were cut from the humeral diaphysis so that the long axes of the blocks are derived from the anterior aspects of the diaphyses of the humeri. Twenty-five blocks were freeze-dried in a Virtis Freeze-Dryer (Biopharma Process Systems, UK) device. The specimens were frozen at -30°C, then vacuumed and finally heated to 15°C for 24 hours to extract the sublimated water from the bone. Nineteen bone blocks were solvent-dehydrated in Tutoplast® Laboratories (Tutogen Medical GmbH, Germany). The specimens were treated with 10% NaCl, acetone, 0.9% NaCl, H_2O_2 and acetone again. Twenty-eight bone blocks were used as controls and did not undergo any procedure.

All specimens were subjected to a three point bending test after being rehydrated in saline for 30 minutes. A bending test was applied by an Instron 1186 universal testing machine (Instron Corp., Canton, MA, USA) with a loading speed of 1 mm/min according to ASTM F417 standard (American Society for Testing Materials, USA).

Strength was calculated according to the following formula:

$$O'_k = (3 \times F \times L) / (2 \times w \times t^2)$$

O'_k : specimen strength
F : force
L : specimen length
w : specimen width
t : specimen height

Specimens underwent analyses for calcium and magnesium contents, using an atomic absorption spectrophotometer (Spectra AA-200) after the mechanical testing. Porcelain crucibles (Halden-Wagner, 101-40) were first heated to 110°C for 1 hour and weighed. 0.05 mg of bone was placed into the holder, heated again to 110°C and measured for weight to calculate the sample bone weight. The samples were than heated in an oven (Heraeus M 11) at 750°C for 1.5 hours. They were then dissolved in 15 ml of concentrated HNO_3, rinsed with distilled water and put in volumetric flasks. Suspension in the porcelain crucible was increased to 100 ml with the addition of distilled water. The samples were further diluted at a ratio of 1:50 with distilled water before being placed in the spectrophotometer by taking 1 ml of the sample and adding distilled water to 50 ml.

All samples were analyzed thrice and the mean of these three values was recorded. Mineral content was calculated using the following formula:

$$X = (X_C \times V \times SF) / M$$

where;
X : mineral content (mg/g)
X_C : value given by the atomic absorption spectrophotometer (mg/l)
V : sample volume (= 100 ml)
DF : dilution factor (= 50)
M : sample weight (g)

One-way ANOVA analysis was applied with SYSTAT 8.0 (SPSS Inc., Chicago, IL, USA) software.

Results

The average strength for the three point bending test was found to be 184.95 MPa and the average calcium content 231.51 mg/g for the freeze-dried group (Figs. 1 and 2). Similarly, the average strength for the three point bending test was 57.55 MPa and the average calcium content was 219.96 mg/g in the solvent-dehydrated group (Figs. 1 and 2). As for the control group, the average strength was 211.85 MPa and the average calcium content was 244.20 mg/g (Figs. 1 and 2).

There was a significant difference between the mechanical strength of the three groups ($p<0.001$). The difference between the freeze-dried, solvent-dehydrated and control groups was found to be statistically significant ($p<0.05$). There was a 12.7% decrease in the freeze-dried group when compared to the control group. The difference between the solvent-dehydrated group and the other two groups was found to be statistically significant ($p<0.05$). There was a 72.84% decrease in the solvent-dehydrated group when compared to the control group (Fig. 1, Table 1).

When compared for calcium content, it was observed that the difference between the solvent-dehydrated and control groups was statistically significant ($p<0.05$), with a decrease of 9.92% (Fig. 2, Table 2). Neither the difference between the freeze-dried and solvent-dehydrated groups nor the difference between the freeze-dried and control groups were statistically significant ($p=0.06$) (Table 2).

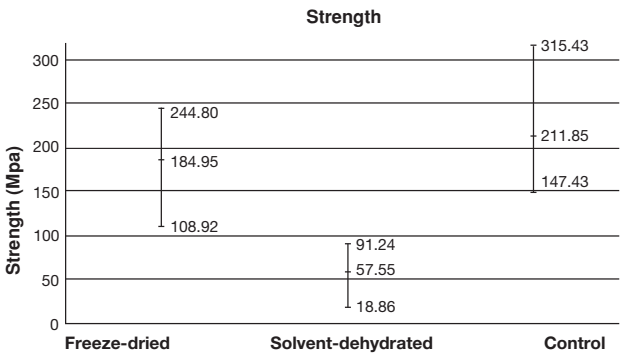

Fig. 1. Strength values for each group.

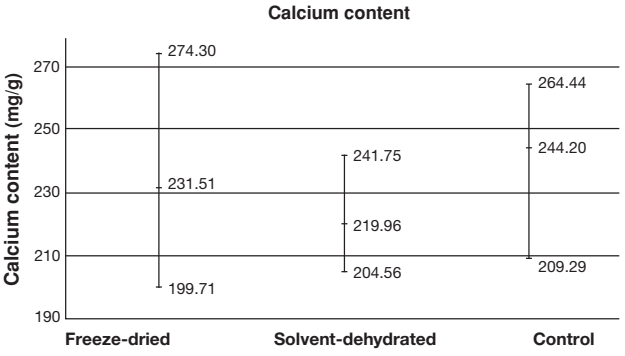

Fig. 2. Calcium content values for each group.

Discussion

Freeze drying, also known as lyophilization, is a method for the preservation of tissue transplants and can increase the shelf life of a bone tissue graft up to 5 years. The tissue is initially deep frozen at -80°C, and then slowly heated and the water content is sublimated. The process ends when the moisture level drops below 5%.[4] It is well known that this method minimally affects the bone while drying.[5] The effects of freeze drying on the mechanical properties of bone have been studied using several different measurement methods.[1,6-9] While some reported a decrease in bone strength,[9,10] others stated that lyophilization did not affect bone strength.[7] It is generally considered that cancellous bone is less affected by lyophilization than cortical bone.[2]

As the symphysis, canine region and anterior body of the mandible are mainly subject to bending forces, we applied a three point bending test to show the decrease in mechanical strength of freeze-dried bone specimens.[6,11]

The results showed a 12.7% decrease in the mechanical strength of freeze-dried specimens compared to that of the control. This corresponded to the findings of Pelker et al.[7] who stated that the bending strength of rehydrated freeze-dried bone decreased by 10 to 45%. It is widely accepted that rehydration of the freeze-dried bone results in a decrease in the mechanical properties.[4,9,10] It has been hypothesized that this decrease in the mechanical strength may be a result of microscopic fractures[12] or chemical changes in the collagen bonds.[6] In this study, while the collagen content was not evaluated, the authors think that the decrease reported in this study may be a result of microscopic formation, as previously reported by Voggenreiter et al.[12]

Mineral content, especially calcium content, is another parameter in determining the mechanical

Table 1. Comparison of the groups for bending strength.

Strength	N	Mean	Standard deviation	Standard error	%95 confidence interval Lowest	%95 confidence interval Highest	Minimum	Maximum
Freeze-dried	25	184.95	28.88	5.78	173.03	196.87	108.92	244.80
Solvent-dehydrated	19	57.55	20.83	4.78	47.51	67.58	18.86	91.24
Control	28	211.85	47.14	8.91	193.58	230.13	147.43	315.43
Total	72	161.79	72.95	8.60	144.65	178.93	18.86	315.43

ANOVA

Strength	Sum of squares	Df	Mean of squares	F	Sig
Between groups	290,057.30	2	145,028.65	113.97	.000
Within groups	87,806.27	69	1,272.56		
Total	377,863.60	71			

properties of bone.[13,14] Current findings in this study show a decrease in the calcium content of freeze-dried specimens. However, this decrease is small, compared to the loss of bending strength in the same specimens. This decrease may be due to changes in the collagen content or integrity of the bone. Garneo et al.[15] have reported that collagen bonds in the bone affect the mechanical properties of bone, independent of mineral content, which is in correlation with the hypothesis mentioned above.

First introduced by Tutoplast® (Tutogen Medical GmbH, Germany), solvent dehydration is a newer preservation method, in which solvents cleanse the tissue of particles and cells.[16] During this process, cells are destroyed with hypertonic salt solutions, viruses and prions are inactivated with hydrogen peroxide, and finally the bone is dehydrated with acetone solutions. Few studies exist on the mechanical properties of solvent-dehydrated bone grafts. Kessler et al. reported a decrease in mechanical strength, but this was related to the sterilization process itself.[17] In the present study, no specimens were sterilized, meaning that the decrease in mechanical strength cannot be related to the sterilization process.

Hydrogen peroxide is one of the agents used in the process of solvent dehydration and releases –OH ions. Hydrogen peroxide is often used in dentistry, and it is believed that –OH ions change the structure of proteins by affecting peptide chains.[18] Also with an acidic pH, hydrogen peroxide is believed to cause porosity in hard tissue.[19] Little research has been made on the effects of hydrogen peroxide on bone. In a study by Moseley et al.,[20] performed on bovine alveolar bone, it was shown that hydrogen peroxide causes alterations in the amino acid compositions and proline levels of proteoglycans. Chng et al.[19] found that a 30% hydrogen peroxide solution significantly decreases the mechanical properties of the dentine tissue.

No publications have been made on the effects of hydrogen peroxide on collagen, which is a very important component of bone for mechanical strength. Acetone, which is used in the dehydration process, has also not been widely investigated for its effects on the mechanical properties of bone. However, it is known that acetone is a highly toxic agent which causes drying and cracking on the skin and mucous membranes with contact.[21]

Hoffmeister et al.[22] also showed that decollagenized bone had lower mechanical properties. In the present study, collagen content was not evaluated.

In our study, the difference in calcium content between the control group and solvent-dehydrated group was statistically significant. The difference between the solvent-dehydrated and freeze-dried groups was not significant. The authors believe that the large decrease in the mechanical strength of the solvent-dehydrated group is not related to its calcium content and further studies on collagen content are required in order to understand the reasons for the detected decrease in the bending strength of solvent-dehydrated bone. Hydrogen peroxide and acetone should also be studied to understand their effects on bone porosity and bone collagen content.

Table 2. Comparison of the groups for calcium content.

Calcium	N	Mean	Standard deviation	Standard error	%95 confidence interval Lowest	%95 confidence interval Highest	Minimum	Maximum
Freeze-dried	25	231.51	19.72	3.94	223.37	239.65	199.71	274.34
Solvent-dehydrated	19	219.96	9.39	2.21	215.29	224.63	204.56	241.75
Control	28	244.20	14.54	3.53	235.58	250.53	209.29	264.44
Toptal	72	231.32	17.89	2.31	226.70	235.94	199.71	274.34

ANOVA

Calcium	Sum of squares	Df	Mean of squares	F	Sig
Between groups	4,667.07	2	2,333.54	9,358	.000
Within groups	14,213.00	57	249.35		
Toptal	18,880.07	59			

Within the limits of the methods used in this study, it was observed that both methods used to dehydrate bone resulted in a decrease in the mechanical properties of the graft material. This should be considered especially in the period prior to osseointegration, in which the mechanical strength of the graft material is important.

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Conflicts of Interest: No conflicts declared.

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