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Biomechanical Comparison of The Effects of The Storage Temperature on Tibiotarsus in Japanese Quail

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Abstract

The study aimed to compare the effects of different cryopreservation temperatures on mechanical properties and determine the optimal cryopreservation temperature for bones in Japanese quail. Bone biomechanical tests are getting more attention but, fresh bones are not always available for testing and have a limited lifespan. Cryopreservation of biological specimens is often needed during tissue preparation and mechanical testing. In the study, the tibiotarsi were collected from 8 weeks of age quail, and bones were divided into four groups of fresh bones; frozen at 0 °C, frozen at -20 °C, and frozen at -80 °C. Frozen bones were kept in the freezer for three weeks. After three weeks, bones were subjected to a three-point bending test for biomechanical evaluation. There was no significant difference between the mechanical strength properties of fresh tibiotarsi and the tibiotarsi stored in three different storage conditions of 0 °C, -20 °C, or -80 °C. It was observed that cryopreservation of tibiotarsi at 0, -20, and -80 °C for up to three weeks did not negatively affect bone biomechanical properties in quail.

Keywords: Biomechanics, cryopreservation, freezing, storage temperature.

Introduction

Over the past decades, bone-breaking strength received more attention. Bone measurements such as bone fracture strength, bone ash, bone ash concentration, bone mineral content, and bone density have been used as crucial indicators of bone status in poultry[1-6]. It is often impractical to test freshly harvested bone[7]. On the other hand, fresh bones are not always available, have a limited lifespan (approx. three weeks), and have difficulties in shipping or handling[8]. Cryopreservation of biological specimens is often unavoidable during tissue preparation and mechanical testing. In particular, it becomes difficult to immediately process bone samples to measure bone strength and other parameters with large numbers of animals. Therefore, if large amounts of bone cannot be processed simultaneously, an effective procedure is required to preserve and stabilize bone mineralization in the bone[9]. Postmortem storage has been used for biomechanical measurement of living tissues, as it is impossible to perform tests in vivo or immediately after sacrifice[10]. Therefore, cryopreservation of tissue samples before mechanical testing has become an accepted technique[11]. However, it is still criticized whether preservation methods affect bone properties or bone strength.
Researchers have conducted several studies to understand the effects of freezing and thawing on bone mechanical properties on various species such as chickens[9], dogs[11], mice[12], rats[13], pigs[14], and deer[15]. In recent years, the use of Japanese quail (Coturnix coturnix japonica) in biomedical research has increased[16], and its use in biological, genetic, aging, and disease research has become widespread[7]. However, it is not known about the effects of freezing at different temperatures on bone properties in quail. Therefore, this experiment aimed to compare the effects of different storage temperatures on some mechanical properties and determine the optimal cryopreservation temperature for future bone biomechanical tests in Japanese quail.

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Material and Methods

Animals and Bone Collection

The tibiotarsi were collected from eight weeks of age quail raised for commercial production (slaughtering material) in Animal Health and Animal Production Research and Application Center in Bursa Uludag University Faculty of Veterinary Medicine. The quail were divided into four groups, and a total of twenty-four quail were used, with six quail in each group. Experimental groups were: fresh bones; frozen at 0 °C (Nuve ES120, NUVE, Ankara, Turkey); frozen at -20 °C (Bosch KSU3921NE/01, Robert Bosch GmbH, Gerlingen, Germany), and frozen at -80 °C (Forma 88000 Series, Thermo Scientific, Massachusetts, United States). After sacrifice, the bones were cleaned surrounding soft tissues. Before freezing, the bones were wrapped in physiological saline (0.9% NaCl) solution-soaked gauze and bagged to prevent drying and tagged[17]. Then, the bones were frozen at different temperatures (0, -20, and -80 °C) until mechanical tests and cortical area analysis were conducted.

Freezing and thawing procedure

1. The fresh group consisted of fresh bones, and mechanical tests were performed at room temperature within 45 minutes after bone collection.
2. The bones in the 0 °C group were stored in sealed plastic bags at 0 oC for 3 weeks, then thawed at 37 °C in an oven (Nuve ES120, NUVE, Ankara, Turkey) for 1h and to be followed by the mechanical test at room temperature.
3. The bones in the 20 °C group were stored in sealed plastic bags at -20 °C for 3 weeks, then they were defrosted at 37 °C in an oven (Nuve ES120, NUVE, Ankara, Turkey) for 1h, and the mechanical test was performed at room temperature after thawing.
4. The bones in the 80 °C group were stored in sealed plastic bags at -80 °C for 3 weeks, then they were defrosted at 37 °C in an oven (Nuve ES120, NUVE, Ankara, Turkey) for 1h, and the mechanical test was performed at room temperature afterward13.

Mechanical Testing

A three-point bending test was performed on each tibiotarsus of quail after the freeze-thaw procedure. A custom-made testing machine, designed by Dr. Kenan Tufekci, according to Tufekci et al.[18], was used to measure force and corresponding displacement for low-strength materials. The tests were performed at a constant loading head speed of 10 mm/min[19]. The average tibiotarsus length was 49.44 ± 1.56 mm; therefore, the span between supports was adjusted to 40% of the total bone length, 20 mm, and the load was applied to the midpoint of the bone length at the middle of the span. The loading was applied with a constant speed of the load head until the bones broke. Ultimate load (Fmax)was read as the highest load from the load-displacement curve. Subsequently, mid-shaft sections of tibiotarsus were obtained from the fracture site with a wire saw, and sections were photographed under a stereomicroscope (Motic, Model: SMZ-168, Hong Kong). Solidworks R17 3D CAD software (Dassault Systèmes, Waltham, MA; USA) was used for determining the cortical area (Acort) and the minimum principal moment of inertia (Imin). Young's modulus or modulus of elasticity (E) was calculated by using the following equation:

E=(FL3)/(48δImin)

Imin is the minimum principal moment of inertia, F is the ultimate load, L is the length between support, δ is the displacement under the corresponding force.

Statistical analysis

As the correlation coefficient for the breaking strength of right and left tibias of broiler chickens is at least 0.90 for fresh bones20, the paired bone technique was used, and left and right tibiotarsi were combined to conduct the statistical analysis. Statistical analyses were performed with IBM SPSS (SPSS, Version 23.0; Chicago, IL). Data were tested for normality of distribution and homogeneity of variances, and one-way analysis of variance (one-way ANOVA) was used for statistical evaluation.

Results

Results of the mechanical three-point bending test and bone properties are presented in Table 1. As a result of three-week freezing, there was no significant difference between the mechanical strength characteristics (ultimate load, minimum moment of inertia, Young's modulus, cortical area) of fresh tibiotarsi and the tibiotarsi stored in three different storage conditions of 0 °C, 20 °C or 80 oC. Likewise, no significant differences were observed between different storage conditions (PFmax=0.657; PImin=0.393; PE=0.731; PAcort= 0.317).

Table 1. Mechanical properties of tibiotarsus measured after different storage methods

	F_{max} (N)	I_{min} (mm^4)	*E* (GPa)	A_{cort} (mm^2)
Fresh	38.268±2.006	1.656±0.131	11.569±0.967	2.865±0.059
0 °C	37.332±1.557	1.646±0.062	11.741±0.577	3.050±0.103
20 °C	40.509±2.012	1.811±0.074	12.124±1.179	3.017±0.077
80 °C	39.248±1.741	1.901±0.175	12.838±0.509	2.924±0.048
P value	0.657	0.393	0.731	0.317

*Data were presented as Mean±SEM (Standart Error of Mean)

*Significance was assessed at the level of P< 0.05.

Fmax: Ultimate load, Imin: Minimum moment of inertia, E: Young's modulus, Acort: Cortical area.

Discussion

Especially in large-scale biomechanical studies, it is not always possible to work with fresh bones immediately after animal sacrifice. Therefore, researchers used several storage methods at different temperatures at various times. Unlike most studies, an avian species, quail, was used instead of a mammalian species in the present study. Park et al.[9] and Lott et al.[21] also investigated the effects of cryopreservation on the mechanical properties of chicken bones. In the present study, quail was selected because its importance has increased in biomedical studies in recent years16. The experiment was designed to examine the effects of freezing at 0°C, -20°C, and -80 oC on some biomechanical properties of the tibiotarsus in quail. This experiment is also the first study comparing the bone biomechanical properties of quail tibiotarsus at different cryopreservation temperatures.

Researchers used different temperatures between -18 °C and -70 °C for various periods before testing specific biomechanical properties of human and animal bones, such as rigidity and elasticity[22] bending, torsion, toughness, and stiffness[23] and ultimate load[24,25]. Based on the previous literature, changes in bone biomechanical properties were assessed with various freezing or cooling storage methods by using compression and bending tests[26]. Literature suggested that mechanical characteristics may be affected by different storage methods, but significant alterations can only be observed in the case of long-term storage[27-31].

In the present study, the effect of various storage temperatures was compared with fresh tibiotarsi and tibiotarsi frozen for three weeks in quail. The results show that there were no significant changes in tibiotarsal mechanical properties in different storage freezing temperatures. Similarly, Nazarian et al.[26] reported that freezing of murine femurs and vertebrae over two weeks did not change the elastic mechanical properties of the femurs. Freezing of human femoral cortical bone specimens[29] and feline humerus and femur[32] did not affect the bone mechanical properties in humans and cats, respectively. Borchers et al.[33] also suggested that freezing to either -20 ºC or -70 ºC did not affect the mechanical properties of trabecular bone in cows. Furthermore, freezing did not affect the mechanical properties of the trabecular bone[27,33-35].

Many researchers reported that freezing the bones for less than five years had no significant adverse effect on bone morphology or function[22-25,33,36]. In contrast to our findings and the studies above, Lott et al.[21] observed only minor alterations in bone strength between fresh and frozen bones at -18 ºC for 48 hours. Lee and Jasiuk[37] also observed that storing the femur at -20°C for five years significantly reduced Young's modulus and ultimate strength. These researchers brought a perspective to this effect and suggested a possible reason for differences in mechanical properties of the bone is the formation and enlargement of ice-crystals[37]. When the bones are frozen at -20 °C, the bones slowly lose moisture due to evaporation[38], which increases the size of the ice crystals and causes structural damage to the bone tissue[39]. Lee and Jasiuk[37] also suggested that cellular enzymes could be another reason for bone degradation. The enzymes that degrade the organic matrix are still effective at -20 °C. To avoid cellular destruction, temperatures of -70 degrees and below should be preferred for long-term storage conditions[37].

In conclusion, it was observed that cryopreservation of tibiotarsi at 0, -20, and -80 °C for up to three weeks did not negatively affect bone biomechanical properties in quail. Therefore, researchers can use 0, -20, and -80 °C temperatures as a convenient preservation method in quail species. In future studies, by planning a study longer than one year, comparing the bone mechanical properties is recommended to examine the long-term effects of cryopreservation temperature.

Conflict of interest

The author declares no conflict of interest.

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