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Original Article

Role of preservation methods using deep-freezing and liquid nitrogen in bone allograft characteristics: An in vitro study

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Abstract

Bone grafting has emerged as a key solution in bone defect management such as allograft, graft of bone from another individual. However, bone allografts usually undergo rigorous preparation to eliminate immune-triggering elements. The deep-freezing methods may delay graft use, while cryopreservation using liquid nitrogen allows rapid freezing but may alter graft characteristics. The aim of this study was to investigate the post-preservation changes in bone allograft characteristics and to compare the effectiveness of deep-freezing and liquid nitrogen methods using animal model. An experimental study using a post-test only control group design was conducted. Fresh-frozen femoral cortical bone was obtained from male New Zealand white rabbits. Preservation by deep-freezing involved placing bone samples in a -80°C freezer for 30 days. For liquid nitrogen preservation, bone grafts were immersed in liquid nitrogen for 20 min, followed by a 15-min rest at room temperature and a final immersion in 0.9% sodium chloride at 30°C for 15 min. Bone samples then underwent evaluation of cell viability, compression, and bending tests. Cell viability test employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and the compression and bending tests used the Universal Testing Machine (UTM). Independent Student t-test or Mann-Whitney U test were used to compare the methods as appropriate. Our study found that the use of deep-freezing and liquid nitrogen resulted in similar outcomes for cell viability, compression, and bending tests, with *p*-values of 0.302, 0.745, and 0.512, respectively. Further exploration with larger sample sizes may help to optimize the methods for specific applications.

Keywords: Bone allograft, deep-freezing, liquid nitrogen, cryopreservation, bone defect

Introduction

The management of bone defects poses a significant challenge in orthopedic cases due to potential complications such as reduced mechanical stability and impaired bone healing. Bone grafting has emerged as a key solution to address bone defects. In the United States alone, bone graft usage reaches 500,000 annually, with a 38% increase in distribution from 2012 to 2015 [1]. Indonesia also sees a rising trend in bone graft utilization, driven by traffic accidents causing bone defects, as evidenced by data from Dr. Soetomo General Academic Hospital, Surabaya, Indonesia [2-6].

Bone defects can be addressed with bone grafts. Allografts are grafts of bone from other individuals preserved through various processes that can be sourced from both living and deceased donors and processed in tissue banks. Concerns arise regarding the potential

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transmission of pathogens from the donor to the recipient, necessitating rigorous sterilization processes for allografts. This may result in a reduction in the osteoinductive capacity of the graft material [7,8]. However, because allografts can be sourced from various individuals, unlike autografts, the quantity of material used is not limited. This enables surgeons to address bone defects over larger areas [9,10].

Preservation methods include a decellularization process that removes immune-triggering materials through chemical and physical methods. A study demonstrated that deep-freezing preservation could minimize cryoinjury but extend the graft preparation time, while cryopreservation with liquid nitrogen offers a rapid freezing alternative [11]. However, it may alter graft characteristics. Comparative studies on the post-preservation characteristics of grafts between deep-freezing and liquid nitrogen methods are essential to understand these changes further [9,12]. The aim of this study was to investigate the changes in the characteristics of the allograft after preservation and compare the two preservation methods, deep-freezing and liquid nitrogen using animal model.

Methods

Study design and sampling

An experimental study utilizing a post-test-only control group design was conducted from August until December 2023. Fresh-frozen cortical bone was obtained from male New Zealand white rabbits (*Oryctolagus cuniculus)* from the Veterinary Hospital of Universitas Airlangga, Surabaya, Indonesia, for preservation through deep-freezing and liquid nitrogen methods. The study consisted of two study groups, each containing four rabbits. Only male New Zealand white rabbits meeting the criteria of being healthy, aged between 6 to 9 months, and weighing 2500 to 3000 grams were used for bone extraction. Rabbits with any signs of illness were excluded from the extraction process.

Animal preparation and acclimation

Eight New Zealand white rabbits were housed in a special room in 50×70 cm cages for one day for acclimatization. The rabbits were fed with pellets at a quantity of 300 grams per day and had access to water *ad libitum*. Rabbit's feces were routinely cleaned every morning. The rabbits were acclimated the following day. Anesthesia was initiated using intramuscular ketamine (25 mg/kg) and intramuscular xylazine (2.5 mg/kg) injected into the thigh muscle. After achieving sedation, euthanasia was performed via a 300 mg intracardiac pentobarbital injection directly into the heart (**Figure 1**).

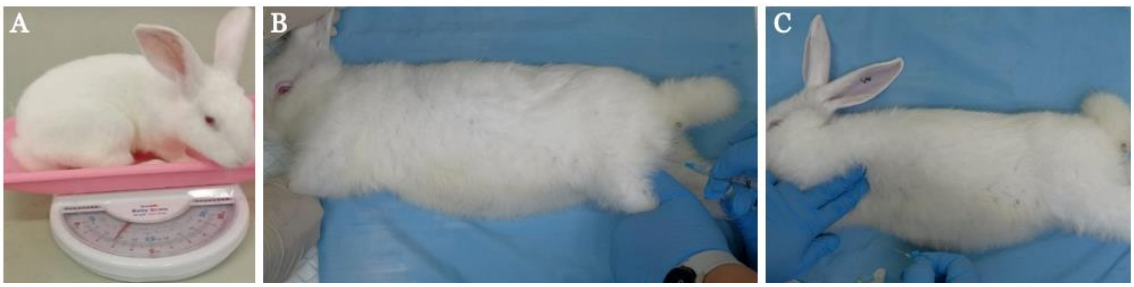

Figure 1. Animal preparation on the New Zealand white rabbit: (A) selection process, (B) anesthesia procedure using intramuscular ketamine and xylazine, (C) acclimation procedure using intracardiac pentobarbital.

Surgical procedures and bone harvesting

The rabbit was placed in a lateral position and the surgical site was shaved using a razor to a distance of 5 cm from the surgical field. The surgical site was cleansed with 0.9% sodium chloride (NaCl), followed by disinfection using 10% povidone-iodine solution. The site was then narrowed with sterile linen. A longitudinal incision was made on the lateral side to harvest the femur bone by disarticulating the femur from the hip joint on the proximal side, and the knee joint on the distal side. Finally, the operating field was closed with a nylon 3.0 thread as recommended [7].

Bone sample collection

The harvested femur bones underwent soft tissue removal and were then measured for length and width. Each rabbit contributed two femur bones: one was prepared for bending tests by cutting the diaphysis 1 cm proximally and distally, while the other was sectioned into equal 1 cm lengths for compression testing (**Figure 2**). Following decellularization, the bone allograft underwent sterilization using 70% ethanol for 10 min, followed by immersion in a 1% solution of antibiotics/antimycotics (consisting of penicillin at 100 IU/mL, streptomycin at 100 mg/mL and amphotericin B at 0.25 ug/mL) for 1 h [8].

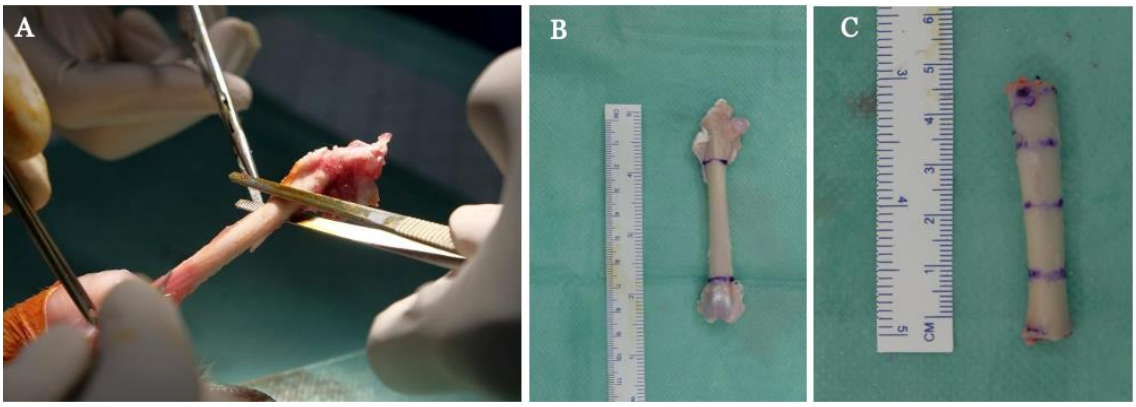

Figure 2. Bone modeling process: (A) removal of soft tissue from the bone; (B) demarcation of the proximal and distal parts of the femur; and (C) demarcation of the femur into sections.

Bone preservation procedure

Bone samples were transported to the Tissue Bank Laboratory of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, with an ice box at a temperature of 4°C for bone preservation procedure. Preservation by deep-freezing was carried out by placing bone samples in a freezer with a temperature of -80°C for 30 days. The samples were put in a plastic wrap using the triple wrap technique (inner layer with polyethylene, middle layer with linen, and outer layer with polyethylene again).

Liquid nitrogen preservation involved immersing the bone graft in liquid nitrogen for 20 min, followed by a resting phase with a 15 min room temperature and a final immersion in 0.9% NaCl at 30°C for 15 min, then the bone graft was packaged using the triple wrap technique and stored at room temperature.

Cell viability test

Cell viability tests were conducted at the Institute of Tropical Disease - Research Center for Vaccine Technology and Development (ITD-RCVTD), Universitas Airlangga, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The bone tissue was placed on a chamber slide and incubated for 8 h. Next, 10 µl of MTT assay reagent was added and incubated for another 3 h until a purple color appeared. Following that, the cells were washed with 100 µl of detergent reagent. The final results were assessed using a fluorescence microscope in the dark and recorded via optical densitometry at an absorbance of 570 nm. The MTT assay evaluated cell viability by measuring color intensity after exposure to MTT. Increased cell viability led to greater conversion of MTT reagent into formazan crystals, yielding higher optical density (OD) readings. Results were expressed in mg/mL units, with lower values indicating reduced cell viability.

Cell viability assessments were conducted on 16 bone samples obtained from rabbits. Each rabbit contributed two bone samples, each measuring 1 cm in length, for this analysis, assuming they could represent broader outcomes. The results of the two bone samples per rabbit were averaged to ensure greater uniformity. Eight bones subjected to the deep-freezing method and another eight subjected to the liquid nitrogen method were placed on chamber slides for examination.

Biomechanical test

Biomechanical tests were carried out at the Department of Mechanical Engineering, Faculty of Industrial Technology and Systems Engineering, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia, using a Universal Testing Machine (UTM) Shimadzu AG-X 5KN (Shimadzu Corp., Kyoto, Japan) for both compression and bending tests (**Figure 3**). The eight bone samples undergoing compression testing were shaped into tube-like structures with a height-to-diameter (H/D) ratio of approximately 2. Samples were selected from previously cut bone samples, with a length of 1 cm and a diameter of 0.5 cm, to ensure optimal strength characteristics without bending, erosion, or double barreling. During the compression test, pressure was applied to the diameter surface, with force was increased at a rate of 10 N/s. The maximum force tolerated, maximum crack displacement, and maximum stress across the entire area were then recorded. In the bending test, pressure was applied to the midpoint of the femur bone incrementally at a rate of 10 N/s. The force and angulation occurring each second were recorded.

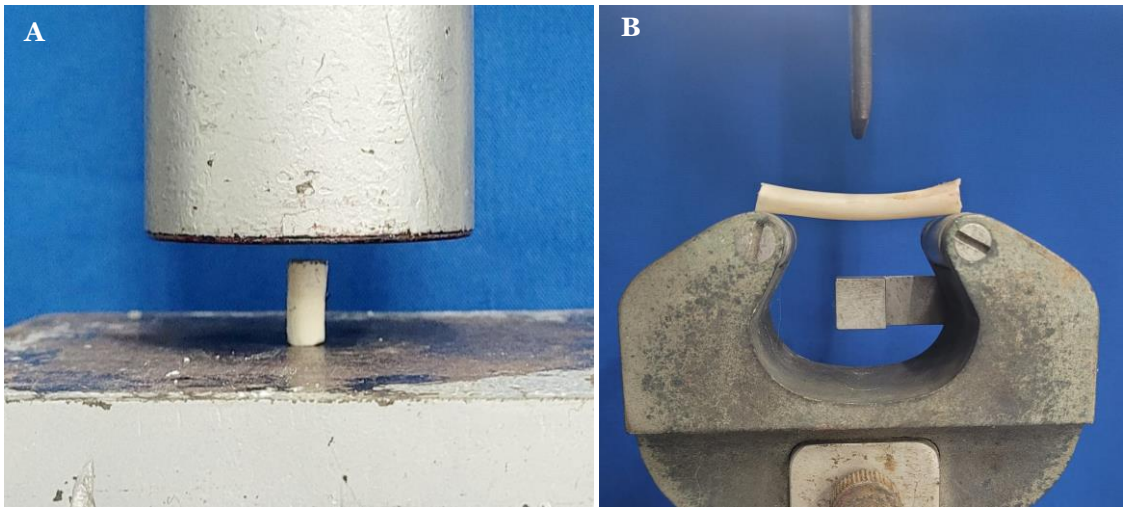

Figure 3. Biomechanical test: (A) compression test, and (B) bending test.

Statistical analysis

The recorded data were categorized, analyzed, and subjected to the Shapiro-Wilk normality test. The independent Student t-test or a Mann-Whitney test was conducted to compare between pre- and post-preservation based on data distribution. All statistical analysis was performed using SPSS 27.0.0 for Windows (SPSS Inc. Chicago, IL, USA).

Results

Cell viability test

The average cell viability of bone samples with preservation methods of deep-freezing and liquid nitrogen were 34.8±2.1 mg/mL and 38.2±8.3 mg/mL, respectively. The comparison analysis between the two methods (deep-freezing and liquid nitrogen) demonstrated no significant differences with p=0.302 (**Table 1**).

Table 1. Comparisons of cell viability of bones preserved using deep-freezing and liquid nitrogen

Preservation method	Rabbit	Viability cell test result (mg/mL)			p-value [a]	p-value [b]
		Bone 1	Bone 2	Mean±SD		
Deep-freezing	1	36.2	36.6	34.8±2.1	0.149	0.302
	2	34.7	34.9			
	3	30.3	34.0			
	4	34.6	37.1			
Liquid nitrogen	5	32.6	31.0	38.2±8.3	0.236	
	6	31.5	28.7			
	7	40.1	48.4			
	8	42.9	50.2			

[a] Analyzed using Shapiro-Wilk test
[b] Analyzed using independent Student t-test

Biomechanical test

The mean compression test results were higher in the deep-freezing group compared to the liquid nitrogen group: the mean maximum force load was 1834.38±513.64 N vs 1692.30±292.53 N; the mean maximum crack displacement was 1.274±0.226 mm vs 0.781±0.681 mm; and the mean maximum stress across all areas was 153.98±28.56 N/mm² vs 147.03±29.06 N/mm². However, the statistical analysis showed no significant difference (p=0.745) between the compression test results for bone preservation using deep-freezing compared to liquid nitrogen (**Table 2**).

Table 2. Compression test results of bones preserved using deep-freezing and liquid nitrogen

Preservation group	Rabbit	Maximum force load (N)	Maximum crack displacement produced (mm)	Maximum stress in all areas (N/mm²)	p-value[a]	p-value[b]
Deep-freezing	1	1466.05	0.935	132.95	0.135	0.745
	2	1441.69	1.370	139.06		
	3	1889.28	1.400	148.12		
	4	2540.50	1.391	195.80		
	Mean±SD	1834.38±513.64	1.274±0.226	153.98±28.56		
Liquid nitrogen	5	1492.01	1.369	146.07	0.833	
	6	1944.79	0.090	108.54		
	7	1390.99	0.302	178.46		
	8	1941.44	1.364	155.06		
	Mean±SD	1692.30±292.53	0.781±0.681	147.03±29.06		

[a] Analyzed using Shapiro-Wilk test
[b] Analyzed using independent Student t-test

On average, the bending test results were also higher in the deep-freezing method compared to liquid nitrogen. The mean maximum force load recorded was 205.52±20.16 N vs 187.68±47.01 N; the mean maximum crack displacement measured was 1.413±0.300 mm vs 1.803 ±0.399 mm; and the mean maximum stress across all areas was calculated at 81.11±12.25 N/mm² vs 72.92±39.41 N/mm². However, the bending test outcomes for bone preservation through deep-freezing did not significantly differ from those with liquid nitrogen, with p=0.512 (**Table 3**).

Table 3. Bending test results of bones preserved using deep-freezing and liquid nitrogen

Preservation group	Rabbit	Maximum force load (N)	Maximum crack displacement produced (mm)	Maximum stress in all areas (N/mm²)	p-value [a]	p-value [b]
Deep freezing	1	227.11	1.806	69.61	0.929	0.512
	2	213.78	1.313	72.00		
	3	179.64	1.088	94.81		
	4	201.55	1.446	88.01		
	Mean±SD	205.52±20.16	1.413±0.300	81.11±12.25		
Liquid nitrogen	5	255.79	1.896	131.61	0.833	
	6	158.90	2.102	47.68		
	7	154.02	1.218	52.90		
	8	182.02	1.999	59.51		
	Mean±SD	187.68±47.01	1.803±0.399	72.92±39.41		

[a] Analyzed using Shapiro-Wilk test
[b] Analyzed using independent Student t-test

Discussion

Autologous bone is often chosen as the go-to graft for treating various bone defects due to its favorable impact on biological healing and the stabilization of surrounding bone structures. Despite attempts to develop better synthetic bone grafts, autologous bone remains the gold standard. Different materials have been explored for reconstructing bones damaged by disease or trauma. Autogenous bone, known for its osteogenic, osteoinductive, and osteoconductive properties, is considered superior for grafting. However, drawbacks include rapid resorption after transplantation, limited preservation space, and the need for additional surgical procedures during harvesting. In contrast, synthetic bone serves as a structural support but lacks significant osteogenic and osteoinductive capabilities [15-17].

Bone grafts fall into three categories: autologous, allograft, and bone substitutes. Autologous bone grafts come in four main forms: cancellous, cortical, corticocancellous, and vascularized.

Surgeons may combine these forms into composite grafts. Autologous bone grafts offer advantages such as cost-effectiveness, ready availability, and easy accessibility. However, drawbacks include donor site morbidity and volume harvesting restrictions [10,15,18]. To address these limitations, allografts undergo a rigorous preparation process, starting with donor selection to ensure disease-free and adequate bone for transplantation. Individuals with osteoporosis or a history of malignancy may be excluded. After passing selection and completing grafting, the allograft undergoes decellularization [12,14]. Desired to remove materials that could trigger an immune response, the decellularization process uses chemical methods (e.g., hydrogen peroxide and enzymes) and physical methods (e.g., centrifugation, sonication, and temperature treatment). A common temperature treatment is the fresh-frozen method, where graft tissues gradually freeze in a deep freezer at -80°C for four weeks. Though preserving biomechanical, osteoinductive, and osteoconductive characteristics, this method has the drawback of a prolonged timeframe from transplantation to graft readiness [19,20]. An alternative method is cryopreservation using liquid nitrogen. This process rapidly freezes tissues by applying a high concentration of cryoprotective agents and exposing them directly to liquid nitrogen. Although quick, this method can alter cellular characteristics due to drastic temperature changes and cryoprotective agent exposure [9,11].

The differences between these two preservation methods are not yet well understood. Discussed in terms of cell viability, a study compared rapid freezing with liquid nitrogen to slow freezing (deep-freezing) for preserving Vero cells. The study concluded that, regardless of the cryoprotectant, deep-freezing seems preferable for Vero cell preservation [21]. Another study also found that frozen valves stored in liquid nitrogen had around 45% viability, with lower viability in deep freezers after two weeks for the mitral valve and four weeks for others [22]. Homografts can be stored in a deep freezer for up to two weeks without deterioration [22].

From a biomechanical and biological perspective, many previous studies have revealed the superiority of liquid nitrogen compared to the freezing method [14,23-25]. A study discovered that autografts treated with liquid nitrogen freezing may result in better osteoinduction outcomes than those treated with extracorporeal irradiation (ECIR) [26]. Another study recommended bone reconstruction with liquid nitrogen-treated bone, emphasizing the free-freezing method [27]. Other studies supported liquid nitrogen as a simple and effective method for biological reconstruction, with positive outcomes in joint-preservation reconstruction [28,29]. Previous studies also concluded, based on in vivo testing, that tumor cells died out with the liquid nitrogen method [26,28-32]. Compression strength analysis showed no significant difference between intact bone and liquid nitrogen-treated bone, while autoclaved bone exhibited decreased strength [30]. In contrast, a different study suggested that the supercooling technique could be an optimal preservation method for cortical bone allografts, based on biomechanical and biological superiority [14].

We extend this discussion of preservation to clinical applications, for example, in the case of tumors. There were some researchers concluded the superiority of liquid nitrogen. A study demonstrated that liquid nitrogen-treated tumor-bearing autografts are effective for biological reconstruction after tumor resection, especially for patients without severe osteolytic bone tumors [33]. Another study also presented cryosurgery with liquid nitrogen as an efficient tool for decreasing the recurrence rate of bone giant cell tumors [31]. They reported no complications in their cases, with good and excellent functional results at a minimum two-year follow-up.

Conclusion

This study found that the effects of the deep-freezing and liquid nitrogen methods on cell viability and compression appear comparable; however, no significant differences were found. Further studies may be necessary to explore additional parameters and optimize preservation methods, especially for more specific applications with larger sample sizes.

Ethics approval

The protocol of the study was approved by the Animal Care and Use Committee (ACUC) of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (No: 2.KEH.136.08.2023).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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