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Comparison of Bone Healing Using Fresh-Frozen Allografts Preserved by Deep-Freezing Versus Liquid Nitrogen Methods

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ABSTRACT

Limb salvage surgery has become the preferred treatment for primary malignant bone tumours, offering functional preservation without compromising oncologic outcomes. Biologic reconstruction using recycled bone allografts processed via deep freezing or liquid nitrogen is increasingly used, particularly in young patients. While both methods aim to maintain structural integrity and osteoconductivity, their effects on bone healing may differ. This study aims to compare the bone healing outcomes of liquid nitrogen-treated and deep-frozen allografts in the context of biologic limb reconstruction. This experimental study employed a post-test control group design involving 16 male New Zealand White rabbits. Animals underwent standardized radial bone defect surgery and were divided into two groups receiving either deep-frozen or liquid nitrogen-preserved bone allografts. Evaluations were conducted histologically, immunohistochemically (VEGF and TGF-β), and radiologically (modified Radiographic Union Scale for Tibial fractures, mRUST) at 4- and 8-weeks post-operation. Comparative analyses demonstrated no significant differences between the two preservation methods regarding osteoblast counts, micro vascularization, VEGF and TGF-β expressions, and mRUST radiological scores. Both methods exhibited similar progression in bone healing with p-values >0,05, respectively. The findings suggest that deep-freezing and liquid nitrogen preservation methods offer equivalent outcomes in supporting bone healing processes. Observed patterns align with known biological phases of bone regeneration, emphasizing comparable efficacy between both methods. Both deep-freezing and liquid nitrogen preservation methods are effective and comparable for bone allograft preservation. Further studies with longer observation periods, biomechanical evaluations, and clinical translational models are recommended to validate these findings

Keywords: *allograft, bone defect, bone graft, deep-freezing, liquid nitrogen*

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1. INTRODUCTION

Primary malignant bone tumours, such as osteosarcoma and Ewing sarcoma, most commonly affect children and young adults. In the past, amputation was the standard treatment for these conditions due to the aggressive nature of the tumours and the lack of effective reconstruction techniques. However, with advances in imaging, chemotherapy, and surgical techniques, limb salvage surgery (LSS) has become a viable alternative, offering patients the possibility of retaining their limbs without compromising oncologic outcomes [1–2].

Limb salvage surgery aims to achieve complete tumour resection with negative margins while preserving the function and appearance of the affected limb. Following wide resection, the resulting bone defect presents a significant reconstructive challenge. Several reconstruction options exist, including endoprosthesis replacement, allografts, autografts, and the use of biological or hybrid methods. Among these, biologic reconstruction has gained increasing attention for its potential to integrate with host tissue, remodel over time, and provide long-term durability [3–5].

Biologic reconstruction techniques include the use of massive bone allografts, vascularized fibular grafts, and recycled autografts processed through methods such as autoclaving, pasteurization, or liquid nitrogen treatment. These techniques offer unique advantages in paediatric and adolescent patients, where skeletal growth and long-term implant survivorship are major considerations. Moreover, biologic grafts may provide superior load-sharing properties and are less likely to require multiple revisions compared to endoprosthesis implants[6-8].

Among the biologic reconstruction options, the use of recycled autografts and allografts has gained popularity, particularly in resource-limited settings or paediatric cases where prosthetic reconstruction poses significant challenges. Two commonly used graft preservation methods are deep-freezing (typically at –80°C) and cryotreatment using liquid nitrogen. Both aim to devitalize tumour cells while maintaining the bone's structural integrity and osteoconductive properties, but their effects on bone healing and tissue response may differ significantly [9–10].

Liquid nitrogen treatment uses rapid freeze-thaw cycles believed to better retain biological matrix and cues than deep freezing, potentially enhancing osteoinduction and neovascularization. However, comparative evidence on healing outcomes between the two methods remains limited, highlighting the need for further objective studies [9-11].

This study aims to evaluate and compare the bone healing process between liquid nitrogen-treated and deep-frozen recycled bone allografts in a non-critical sized defect model. Healing will be assessed histologically, immunohistochemically, and radiologically at defined intervals, providing multidimensional evidence on which preservation method better supports biologic integration in the context of limb salvage reconstruction [11-12].

2. MATERIAL AND METHODS

2.1 Study Design

This study was conducted as pure experimental laboratory research using a post-test only control group design. The objective was to compare bone healing between deep-frozen and liquid nitrogen–preserved allografts in a New Zealand White rabbit radius non-critical sized bone defect model. The experimental subjects were male New Zealand White rabbits maintained under controlled housing and feeding conditions. The inclusion criteria were healthy male rabbits, aged 4–5 months, with no physical deformities, exhibiting active movement, and weighing between 2500 and 3000 grams. Rabbits that became ill or died during the experimental period were excluded from the study.

Ethical Procedure

All experimental procedures involving animals were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Veterinary Medicine, Airlangga University, under ethical clearance number 2.KEH.140.10.2024.

2.3 Sample Size and Group Allocation

Sample size was calculated using a standard formula based on prior data, with a standard deviation of 3, Zα of 1.96 ($\alpha = 0.05$), and Zβ of 1.84 ($\beta = 0.10$). The calculation yielded a requirement of 3.5 animals per group, which was rounded to 4. Thus, a total of 16 rabbits were randomly divided into four groups: two groups received deep-frozen allografts evaluated at weeks 4 and 8, while the remaining two groups received liquid nitrogen–preserved allografts evaluated at the same time points.

2.4 Graft Preparation

Bone allografts were harvested from donor New Zealand White rabbits and preserved using two methods. In the deep-

freezing method, bone samples were wrapped using a triple wrap technique consisting of polyethylene as the inner layer, linen as the middle layer, and polyethylene as the outer layer. Samples were stored in an ice box at 4°C and subsequently preserved at −80°C for 30 days in the deep freezer at the Tissue Bank Laboratory. In the liquid nitrogen method, the bone grafts were immersed in liquid nitrogen for 20 minutes, left at room temperature for another 20 minutes, and finally soaked in 0.9% NaCl solution at 25°C for 20 minutes. These samples were also wrapped using the triple wrap technique.

2.5 Acclimatization

All animals underwent a one-week acclimatization period in the animal housing facility of the Faculty of Veterinary Medicine, Universitas Airlangga. Any animal found to be sick during this period was excluded from the study.

2.6 Anaesthesia and Surgical Procedure

Anaesthesia was induced using intramuscular injections of ketamine (40 mg/kg) and xylazine (13 mg/kg), with onset in 3 to 6 minutes. Additional maintenance doses of ketamine (10 mg/kg IM) were given as needed. Rabbits were fasted for six hours postoperatively.

The anterior surface of the rabbit radius was shaved and disinfected with 10% povidone iodine. A 1.5 cm incision was made to expose the mid-diaphysis, where a cylindrical bone defect measuring 1 cm in diameter and 2.5 mm deep was created using a 2.5 mm drill, penetrating the medullary canal. The defect was filled with the assigned allograft. Muscle and skin were sutured with absorbable 3-0 sutures, and the surgical site was covered with a waterproof dressing. Intramuscular cefazolin was given during the procedure and continued for three days postoperatively.

2.7 Euthanasia and Sample Collection

At the fourth- and eighth-weeks post-surgery, animals were euthanized via intraperitoneal injection of pentobarbital at a dose of 60–100 mg/kg. The radius was disarticulated at the elbow and harvested for evaluation. Radiographic imaging was performed to assess union at the defect site.

2.8 Histological and Immunohistochemical Evaluation

The harvested radius specimens were fixed in 70% formaldehyde for seven days, decalcified in 20% formic acid for six days, and embedded in paraffin. Axial tissue sections with a thickness of 5 μm were prepared, and at least four samples were collected per radius. Haematoxylin–eosin (HE) staining was used to evaluate osteoblast count and micro vascularization. Immunohistochemical staining was performed to assess VEGF and TGF-β expression. All microscopic evaluations were performed independently by two observers using a Carl-Zeiss light microscope.

2.9 Statistical Analysis

The collected data were expressed as mean and standard deviation. The Kolmogorov–Smirnov test was used to assess the normality of data distribution. If the data were normally distributed ($p > 0.05$), comparisons between groups were analysed using the independent samples t-test. If the data did not meet the assumption of normality ($p < 0.05$), the Mann–Whitney U test was used as an alternative. The selection of statistical tests was based strictly on normality test results to ensure validity and reliability of the analysis.

3. RESULTS

3.1 Histological Evaluation

3.1.1 Osteoblast

The impact of cryopreservation method on osteoblast viability was assessed by quantifying osteoblasts within the graft tissue following preservation via deep-freezing and liquid nitrogen, evaluated at weeks 4 and 8. At 4 weeks, the mean osteoblast count in the liquid nitrogen group was 81.50 ± 20.07, compared to 68.75 ± 20.27 in the deep-freezing group. Statistical analysis using the Mann–Whitney U test demonstrated a near-significant difference ($p < 0.07$), indicating a trend toward higher osteoblast viability in the liquid nitrogen group, although this did not reach the threshold for statistical significance ($p < 0.05$). At 8 weeks, osteoblast counts declined in both groups, with means of 48.50 ± 31.88 and 43.23 ± 29.21 for the liquid nitrogen and deep-freezing groups, respectively. The observed difference was not statistically significant ($p < 0.250$). These findings suggest that cryopreservation using liquid nitrogen may better preserve osteoblast viability at earlier time points, though the effect was not sustained nor statistically significant at 8 weeks (Table 1; Figure 1).

Table 1. Comparison of Osteoblasts in Liquid Nitrogen and Deep-Freezing Bone Graft

Variable	Sum of Osteoblast (Mean ± SD)	p-Value
4-week Liquid Nitrogen	81,50 ± 20,07	< 0.07*
4-week Deep-freezing	68,75 ± 20,273	
8-week Liquid Nitrogen	48,50 ± 31,876	< 0.250*
8-week Deep-freezing	43,23 ± 29,205	

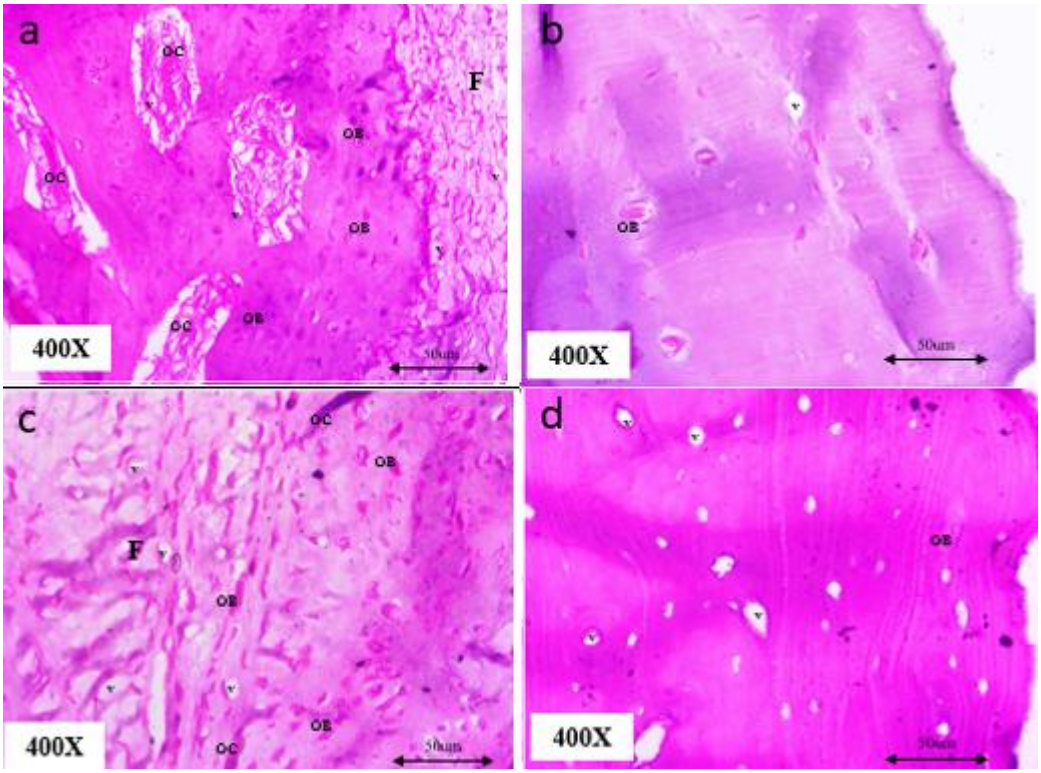

Figure 1. Haematoxylin and Eosin evaluation in the liquid nitrogen a) in week-4; b) in week 8; Haematoxylin and Eosin evaluation in deep-freezing c) in week 4; d) in week 8

3.1.2. Micro vascularization

Microvascular density within the grafted tissue was also evaluated histologically. At week 4, the liquid nitrogen group demonstrated a mean micro vascularization score of 60.30 ± 23.87, whereas the deep-freezing group exhibited a higher mean score of 68.10 ± 16.74. The Mann–Whitney U test yielded a p-value of 0.076, suggesting a potential difference that did not achieve statistical significance. By week 8, the mean scores had decreased to 45.57 ± 16.62 in the liquid nitrogen group and 43.95 ± 12.60 in the deep-freezing group, with no statistically significant difference observed (p = 0.992). These results indicate that while early microvascular response may favor deep-freezing, the differences between preservation methods were not significant at either time point (Table 2; Figure 1).

Table 2. Comparison of Micro vascularization in Liquid Nitrogen and Deep-Freezing Bone Graft

Variable	Micro vascularization (Mean ± SD)	p-Value
4-week Liquid Nitrogen	60,30 ± 23,87	0.076
4-week Deep-freezing	68,10 ± 16,74	
8-week Liquid Nitrogen	45,57 ± 16,62	0.992
8-week Deep-freezing	43,95 ± 12,60	

Immunohistochemical Evaluation of VEGF and TGF-β

VEGF Expression

VEGF expression, as a marker of angiogenic activity, was evaluated at both 4 and 8 weeks. At week 4, the mean VEGF concentration was 4.95 ± 1.98 in the liquid nitrogen group and 5.63 ± 2.23 in the deep-freezing group, with a non-significant difference (p = 0.110). At week 8, the mean values were 3.80 ± 0.99 for the liquid nitrogen group and 4.00 ± 1.34 for the deep-freezing group, with a p-value of 0.922. These results suggest that the method of cryopreservation did not significantly affect VEGF expression during the observed healing periods (Table 3; Figure 2).

Table 3. Comparison of VEGF Concentration in Liquid Nitrogen and Deep-Freezing Bone Graft

Variable	VEGF Concentration (Mean ± SD)	p-Value
4-week Liquid Nitrogen	4,95 ± 1,98	0.110
4-week Deep-freezing	5,63 ± 2,23	
8-week Liquid Nitrogen	3,80 ± 0,99	0.922
8-week Deep-freezing	4,00 ± 1,34	

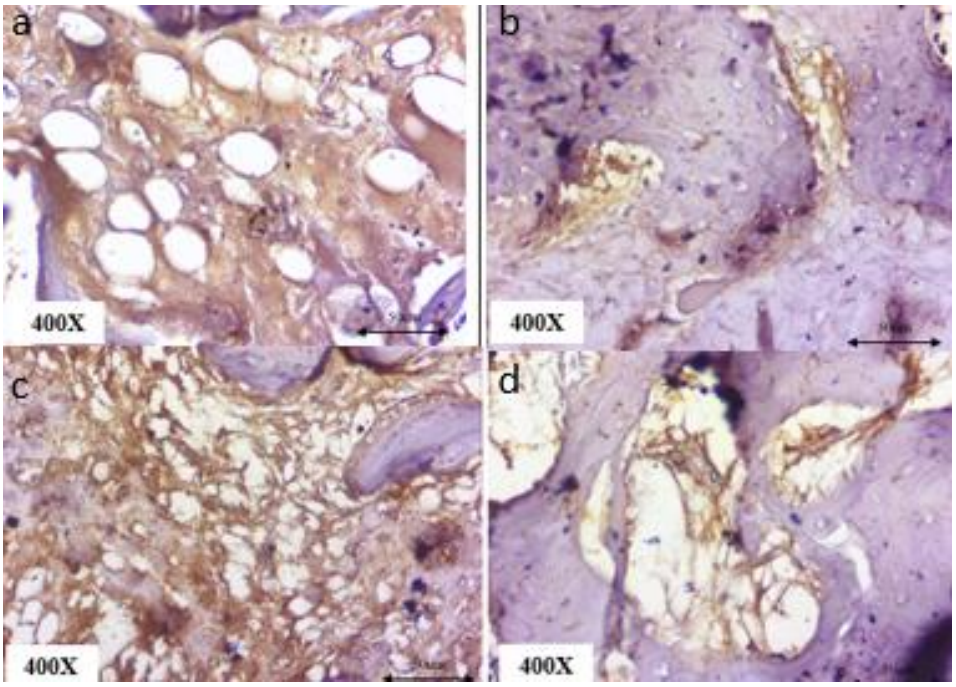

Figure 2. Immunohistochemistry Evaluation of VEGF in the liquid nitrogen a) in week-4; b) in week 8; Haematoxylin and Eosin evaluation in deep-freezing c) in week 4; d) in week 8

TGF-β Expression

TGF-β concentration was assessed to evaluate growth factor stability within preserved grafts. At 4 weeks, the mean TGF-β level in the liquid nitrogen group was 5.28 ± 1.88, while the deep-freezing group showed a higher mean of 6.03 ± 2.16 (p = 0.100). At 8 weeks, TGF-β levels were similar between groups, with means of 5.10 ± 1.69 for the liquid nitrogen group and 5.18 ± 0.84 for the deep-freezing group (p = 0.332). No statistically significant differences were observed at either time point, indicating comparable preservation of TGF-β expression between the two methods (Table 4; Figure 3).

Table 4. Comparison of TGF-β in Liquid Nitrogen and Deep-Freezing Bone Graft

Variable	TGF-β Concentration (Mean ± SD)	p-Value
4-week Liquid Nitrogen	5,28 ± 1,88	0.100
4-week Deep-freezing	6,03 ± 2,16	

8-week Liquid Nitrogen	5,10 ± 1,69	0.332
8-week Deep-freezing	5,18 ± 0,844	

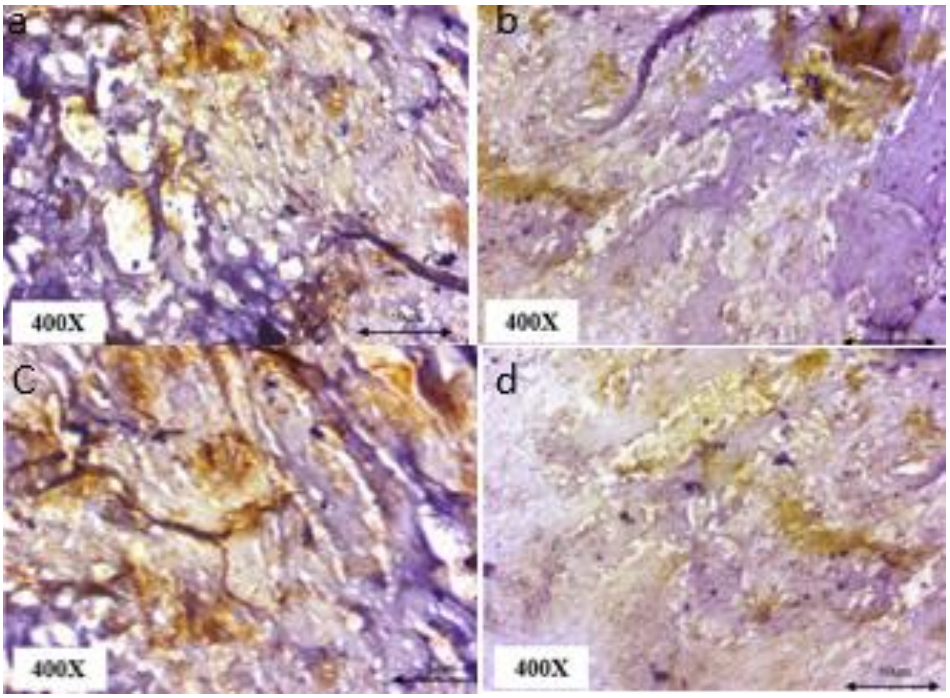

Figure 3. Immunohistochemistry Evaluation of TGF-β in the liquid nitrogen a) in week-4; b) in week 8; Haematoxylin and Eosin evaluation in deep-freezing c) in week 4; d) in week 8

Radiological Evaluation

The modified Radiological Union Scale for Tibia (mRUST) score. Bone healing was assessed radiologically using the modified Radiological Union Scale for Tibia (mRUST). At 4 weeks, the mean mRUST score was 4.50 ± 0.289 in the liquid nitrogen group and 4.25 ± 0.50 in the deep-freezing group, with no statistically significant difference (p = 0.495). At 8 weeks, the mean mRUST score increased to 7.00 ± 0.707 in the liquid nitrogen group and 6.25 ± 0.479 in the deep-freezing group, again without statistical significance (p = 0.343). These findings suggest progressive radiological union in both groups over time, with a non-significant trend toward improved healing in the liquid nitrogen group (Table 5; Figure 4).

Table 5. Comparison of mRUST Score in Liquid Nitrogen and Deep-Freezing Bone Graft		
Variable	mRUST Score (Mean ± SD)	p-Value
4-week Liquid Nitrogen	4.50 ± 0.289	0.495
4-week Deep-freezing	4.25 ± 0.500	
8-week Liquid Nitrogen	7.00 ± 0.707	0.343
8-week Deep-freezing	6.25 ± 0.479	

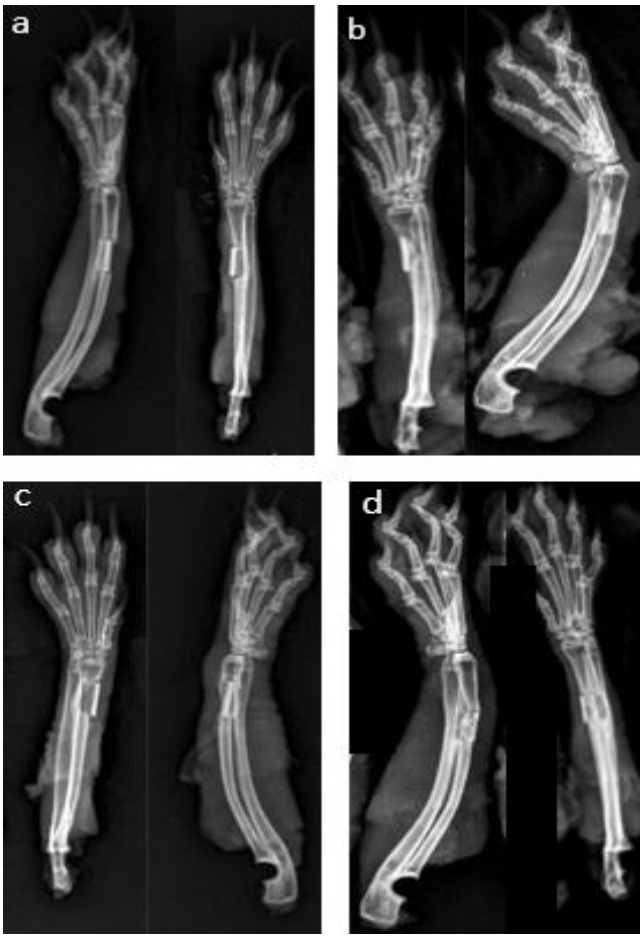

Figure 4. Radiology Evaluation in the liquid nitrogen a) in week-4; b) in week 8; Haematoxylin and Eosin evaluation in deep-freezing c) in week 4; d) in week 8

4. DISCUSSION

Limb salvage surgery has replaced amputation as the standard for treating malignant bone tumours in young patients. Biologic reconstruction, using allografts or recycled autografts, offers long-term durability and host integration. Methods such as liquid nitrogen or deep-freezing are commonly used to prepare grafts, especially in paediatric or resource-limited settings, where prostheses may be less suitable [1-3,5–6, 9-10].

Liquid nitrogen preserves bone matrix and biological cues better than deep freezing, potentially enhancing osteoinduction and vascularization. However, direct comparisons between both methods remain limited. Prior in vitro studies demonstrated that these grafts possess adequate biomechanical strength and are non-toxic [9-10]. The current study further examined these findings using in vivo histological, immunohistochemical (IHC), and radiological evaluations.

At week 4, histological results showed increased osteoblast count and microvascular network formation, supported by elevated VEGF and TGF-β expressions indicating active angiogenesis and osteoblast differentiation [13-15]. Radiological analysis complemented these findings, demonstrating early callus formation and initial bone fragment union. By week 8, the osteoblast count decreased as expected due to differentiation into osteocytes, consistent with typical bone maturation.[16,17] Concurrently, microvascular networks became more organized, VEGF expression decreased, and TGF-β levels remained elevated, indicating a transition to bone remodelling and maturation stages.[18,19]

VEGF concentration showed a positive, though non-significant, correlation with micro vascularization at both time points, suggesting VEGF's crucial role in early angiogenesis. TGF-β concentrations peaked at week 4 and declined by week 8, aligning with its role in initial osteoblast activity and subsequent reduction during bone matrix mineralization and remodelling phases.[16-19]

Radiological evaluation using modified Radiographic Union Scale for Tibial fractures (mRUST) indicated that bridging callus formation was evident in both groups by week 8, suggesting progression to advanced bone remodelling. Although scores were slightly higher in the liquid nitrogen group, statistical analysis showed no significant differences, confirming both preservation methods' comparable efficacy in bone healing.[20]

5. CONCLUSIONS

Bone healing outcomes of allografts preserved via deep-freezing and liquid nitrogen methods demonstrated equally effective results, with no significant differences observed between these two rapid freezing preservation techniques. Further studies with extended observation periods (≥12 weeks), biomechanical evaluations, and use of animal models more closely resembling human conditions or initial clinical trials are recommended to comprehensively evaluate long-term graft integration and validate the clinical applicability of these preservation methods.

6. ACKNOWLEDGEMENTS

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Authors' Contribution

Conceptualization: Muhammad Dimas Arya Candra Permana, Ferdiansyah Mahyudin, Mouli Edward. Data curation: Muhammad Hardian Basuki, Yunus Abdul Bari, Muhammad Phetrus Johan. Formal analysis: Muhammad Dimas Arya Candra Permana, Ferdiansyah Mahyudin, Mouli Edward. Funding acquisition: Muhammad Dimas Arya Candra Permana. Investigation: Muhammad Dimas Arya Candra Permana, Ferdiansyah Mahyudin, Mouli Edward. Methodology: Ferdiansyah Mahyudin, Mouli Edward, Muhammad Hardian Basuki. Project administration: Ferdiansyah Mahyudin, Mouli Edward. Resources: Muhammad Dimas Arya Candra Permana. Supervision: Muhammad Hardian Basuki, Yunus Abdul Bari, Muhammad Phetrus Johan. Validation: Muhammad Hardian Basuki, Yunus Abdul Bari, Muhammad Phetrus Johan. Writing-original draft: Muhammad Dimas Arya Candra Permana, Ferdiansyah Mahyudin, Mouli Edward. Writing-review & editing: Muhammad Hardian Basuki, Yunus Abdul Bari, Muhammad Phetrus Johan.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request

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