European Journal of Medical and Health Sciences
www.ejmed.org

Differences in Cell Death and BMP-2 Expression in Core Biopsy Specimens of Malignant Bone Tumors Given Hydrogen Peroxide Compared to Liquid Nitrogen

Agung Kenji Arnaya, I Ketut Suyasa, I Gede Eka Wiratnaya, and I Wayan Juli Sumadi

ABSTRACT

Introduction: **Malignant bone tumors have a global prevalence of 1% of the overall malignancy case, with a very low 5-year survival rate and high local recurrence. The limb-salvage surgery with bone recycling is widely developed in the management of malignant bone tumors. Hydrogen peroxide and liquid nitrogen are bone recycling mediator agents destroying the malignant tumor cells while preserving healthy bones as much as possible. The purpose of this study was to compare the effectiveness of hydrogen peroxide and liquid nitrogen with the number of tumor cell deaths using the Huvos score and BMP-2 expression in malignant bone tumors.**

Materials and Method: **In vitro experimental research was performed on 30 core needle biopsy samples of bone malignant tumors divided into 2 groups of hydrogen peroxide and liquid nitrogen. The parameters measured are Huvos score and BMP-2 level.**

Results: **Based on this study, the characteristics of malignant bone tumors were dominated by primary tumors (83.9%), in female patients (53.3%) with an average age of 30 years. Liquid nitrogen gave better results than hydrogen peroxide, marked by a lower Huvos score (average 12.13 vs. 18.87, p=0.033) and a higher BMP-2 (average 39.53 ± 26.59 vs. 63.87 ± 27.61, p=0.020).**

Conclusion: **Liquid nitrogen is an effective bone recycling agent for the management of malignant bone tumors.**

Keywords: Hydrogen peroxide, liquid nitrogen, limb salvage, malignant bone tumor.

Submitted : June 3, 2023

Published : July 4, 2023

ISSN: 2593-8339

DOI: 10.24018/ejmed.2023.5.4.1840

A. K. Arnaya*
Resident, Orthopedic and Traumatology Department, Faculty of Medicine, Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital, Denpasar, Bali, Indonesia.
(e-mail: scvngr28@gmail.com)
I. K. Suyasa
Consultant, Orthopedic and Traumatology Department, Faculty of Medicine Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital, Denpasar, Bali, Indonesia.
I. G. E. Wiratnaya
Consultant, Orthopedic and Traumatology Department, Faculty of Medicine Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital, Denpasar, Bali, Indonesia.
I. W. J. Sumadi
Consultant, Anatomy Pathology Department, Faculty of Medicine Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital, Denpasar, Bali, Indonesia.

Corresponding Author

I. Introduction

Epidemiologically, bone and tissue tumors are rare and only occupy 1% of all cancer diagnoses [1]. However, patients with osteosarcoma, especially in long bones, have a low 5-year survival rate of around 29.8% in patients > 60 years of age [2]. Along with the high local recurrence of malignant bone tumors, new treatments for malignant bone tumors have been developed. One of the newest approaches is limb-salvage surgery. As in this approach, agents are used to induce tumor cell death such as hydrogen peroxide and liquid nitrogen. Tumor cell death is an expected condition of bone malignant tumor therapy. The method that can be used is by bone recycling using hydrogen peroxide and liquid nitrogen material. Hydrogen peroxide works by changing the integrity of the membrane and DNA from cells and has an effect in maintaining osteoinductive in the bones. Liquid Nitrogen lysis the tumor cell wall so that it can cause tumor cell death [3]. One way to determine the death of tumor cells is to use the Huvos score and also as a predictor of the success of chemotherapy [4]. Besides the use of histopathological methods, the assessment of bone morphogenetic protein-2 (BMP-2) signaling also has an important role as a predictor of osteogenic properties. BMP-2 also has an important role in the induction of new bone formation and repair [5]. Therefore, this study wanted to examine the differences in cell death as measured by huvos score and BMP 2 expression in core biopsy specimens of malignant bone tumors given hydrogen peroxide compared to liquid nitrogen. Currently there is still minimal literature and basic research related to the comparison of giving hydrogen peroxide compared to liquid nitrogen. Moreover, it is hoped that this research could develop the limb-salvage surgery technique, especially with

hydrogen peroxide and liquid nitrogen in such a way that it can increase the survival rate of patients with malignant bone tumors.

II. Materials and Methods

This research was an invitro experimental study. The research began in July 2022 until November 2022 after obtaining a statement of ethical clearance from the Research Ethics Commission of the Faculty of Medicine, Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital Denpasar through Letter Number 1227 / UN14.2.2.VII.14 / LT / 2022 dated July 7th, 2022.

The samples were 15 patients suffering from malignancy of the long bones at Prof. dr. I.G.N.G. Ngoerah General Hospital, who met the inclusion and exclusion criterias set by the researcher and the samples were taken by consecutive sampling. The sample's inclusion criteria were 1) All patients with malignant bone tumors underwent core biopsy 2) Patients agreed to be sampled in this study. The sample's exclusion criteria were 1) Patients with metabolic and autoimmune diseases of the bones 2) Patients refused to participate in the study 3) Not included in the acceptable criteria for operative therapy. The drop-out's criteria were 1) Patients who withdraw from being research subjects before the deadline is over 2) Samples are damaged so that the Huvos Score and BMP cannot be assessed.

Researcher performed bone tumor biopsies in patients with post-chemotherapy malignant bone tumors who had previously obtained informed consent. Biopsy samples were divided into groups with liquid nitrogen and groups with hydrogen peroxide. Samples were stored in a sterile place and processed with hydrogen peroxide and liquid nitrogen. The liquid nitrogen procedure was carried out by taking a bone tumor and then freezing it in liquid nitrogen for 20 minutes. Then, researcher let it at room temperature for 15 minutes and immersed it in water for 10 minutes. The hydrogen peroxide procedure was carried out by inserting the sample into the STERRAD sterilizer which had been injected with a 58% hydrogen peroxide solution.

An analysis was performed on the huvos score and expression of BMP-2 between the group with liquid nitrogen and the group using hydrogen peroxide. Huvos score was an instrument used to measure the degree of necrosis of cells after receiving therapy. The percentage could be measured according to table I. BMP-2 expression was the result of measuring the expression of BMP-2 protein in tumor cells using the IHC method. The expression of this measurement used an immune score system by calculating positive results compared to controls with the condition of the percentage of positive tumor cells seen in five large visual fields. Positive results showed the same color as the control.

Descriptive analysis was carried out for data on patient characteristics such as sex and age of the patient which was presented in frequency data. Besides, this research used Mann Whitney test to analyze the Huvos score and independent t test to analyze the levels of BMP-2. The significance level (α) of this study is set at a probability value (p) of less than 0.05. All statistical analyzes were performed using the Statistical Package for the Social Sciences (SPSS) for Windows Version 21.

TABLE I: Huvos Score

Grade	Histology
I	Minimal or no necrosis (0-50%).
II	Areas of acellular tumor osteoid, necrotic, or fibrotic material attributable to the effect of chemotherapy, with other areas of histologically viable tumor (Necrosis 51-90%).
III	Predominant areas of acellular osteoid, necrotic, or fibrotic material attributable to the effect of chemotherapy, with only scattered foci of histologically viable tumor cells identified (Necrosis 91%-99%).
IV	No histologic evidence of viable tumor identified within the entire specimen (Necrosis 100%)

III. Results

Descriptive analysis showed more female patients as many as 16 patients (53.3%) than male patients as many as 14 patients (46.6%). The mean and standard deviation for age in this study were 30 ± 20.2 with a p value of 1.0. From the type of tumor, primary tumors were found in 26 samples (83.9%) and secondary tumors were 4 samples (12.9%) with a p value of 1.0. The characteristics distribution of subjects can be seen in Table II.

TABLE II: The Characteristic Distribution of The Research Subjects

Variable	n (%)	Mean ± Standard Deviation	P value (P <0,05)
Age (year)	30	30 ± 20,2	1,0
Sex			
Male	14 (46,6%)	-	1,0
Female	16 (53,3%)	-	
Type of tumor			
Primary tumor	26 (83,9%)	-	1,0
Secondary tumor	4 (12,9%)	-	

A normality test was conducted with the Saphiro-Wilk test because the samples were less than 50. The normality test aimed to find out whether the research data is normally distributed or not. Based on the normality test on the Huvos Score and BMP-2 data, the Huvos Score variable was not normally distributed with p value = 0,000, whereas in the BMP-2 variable found normally distributed with p value = 0.065. Normality test's result on the Huvos Score and BMP-2 could be seen in Table III.

TABLE III: Normality Test on The Huvos Score and BMP-2

Variable	N	P	Results
Huvos Score	15	< 0.05	Abnormal distribution
BMP-2	15	0.065	Normal distribution

Based on the normality test, the Huvos Score data distribution is not normal. Thus, data analysis of the Huvos Score variable was carried out using the Mann-Whitney test. The difference between the Huvos Score in the core biopsy specimen of the malignant bone tumor given hydrogen peroxide compared to liquid nitrogen could be seen in Table IV. Based on the analysis, it was found a higher Huvos Score group in the liquid nitrogen group with significant p value = 0.033.

Based on the normality test, a normal BMP-2 data distribution was analyzed using independent T test. The difference between BMP-2 in core biopsy specimens of malignant bone tumors given hydrogen peroxide compared to

liquid nitrogen could be seen in Table V. Based on the independent t test in BMP 2 levels in the hydrogen peroxide and liquid nitrogen group, BMP 2 levels were found higher in the hydrogen peroxide group with an average difference of 24.3 and statistically significant with p value = 0.020.

TABLE IV: NORMALITY TEST ON THE HUVOS SCORE AND BMP-2

Variable	Mean		P value (P <0,05)
	Hydrogen Peroxide	Liquid nitrogen	
Huvos Score	12,13	18,87	0,033

TABLE V: COMPARISON OF BMP-2 IN CORE BIOPSY SPECIMENS OF MALIGNANT BONE TUMORS GIVEN HYDROGEN PEROXIDE COMPARED TO LIQUID NITROGEN

Variable	Mean ± Standard Deviation		P value (P <0,05)
	Hydrogen Peroxide	Liquid nitrogen	
BMP 2	63,87 ± 27,61	39,53 ± 26,59	0,020

IV. DISCUSSION

From the descriptive analysis, it was found that the characteristics of female patients were greater as many as 16 samples (53.3%), while male patients were 14 samples (46.6%). This was in accordance with a global survey by [2] which showed that the ratio of affected women to men was 1.43:1. In addition, other studies also supported that gender was related to the incidence of osteosarcoma where the incidence rate in women especially at the age of 0-14 years was higher [6]. This is due to the influence of the estrogen. The estrogen plays an important role in osteoclast replication so that mutations in the estrogen receptor can disrupt the balance of bone formation and absorption [7].

The age peak for osteosarcoma itself is bimodal. The first peak occurs at the age of 0-14 years which is related to bone growth. The second peak is at the age of more than 65 years as metastases from other malignancies. In this study, an average age of 30 ± 20.2 years was obtained which did not follow a bimodal pattern of patient age distribution. According to [8], osteosarcoma was a rare tumor that could be found in all ages. In several types of osteosarcomas, there was a predominance of different ages. For example, in parosteal osteosarcoma, it was more often found in young adults around 20-40 years [8]. Likewise according to other study, it was found that osteosarcoma was most commonly diagnosed at the age of 1-30 years [1]. In addition, in cases of Ewing sarcoma, the peak incidence was between 10 and 15 years of age, with approximately 30% of cases occurring in children under 10 years of age, and another 30% in adults over 20 years of age with a ratio of three to one with female more dominant [9]. Furthermore, the incidence of bone metastases was also a concern of this study. In the latest study by [10], out of 151 patients, the incidence of bone metastases was found to be 51% in men and 49% in women. The highest incidence occured between the ages of 50-59 years.

In this study, the average age of 30 years was estimated because osteosarcoma that met the inclusion criteria was not specific to only one type of osteosarcoma. The age distribution of patients with an average of 30 years represented young adult patients who came to the hospital in this study. Patient variations were not in accordance with the clinical conditions in the community.

In this study, a comparison was made of the number of tumor cell deaths in core biopsy specimens of malignant bone tumors between hydrogen peroxide and liquid nitrogen as assessed by the Huvos score, where it was found that the liquid nitrogen group had a higher Huvos score than hydrogen peroxide with an average difference of 6. 74. This value was declared statistically significant (p value = 0.033). Huvos score describes the amount of tumor cell death. This shows that the number of cell death was better with the use of liquid nitrogen than with hydrogen peroxide.

Liquid nitrogen has the ability to freeze faster in larger areas of necrosis. The speed affects the prognosis because when the freezing process is slow it will actually preserve tumor cells. Meanwhile, what we hope from the process is the induction of tumor cell death. Liquid nitrogen can damage the osmolarity of cells by forming ice crystals which trigger apoptosis [11]. According to research by [12], bone recycling with liquid nitrogen for reconstruction after resection of malignant bone tumors offers many advantages. Of the 4 cases of bone recycling with liquid nitrogen, the average Musculoskeletal Tumor Society (MSTS) functional score was 75% without infection and local recurrence. Reconstruction using liquid nitrogen provides good local control and functional results [12].

Research by [13], showed a decreased potential for tumor cell proliferation in vivo by using the liquid nitrogen method by immunostaining bromodeoxyuridine (BrdU). On the other hand, according to [14], hydrogen peroxide had efficacy value as an adjuvant therapy after local curettage for bone tumors. Study by [15], which stated that hydrogen peroxide induces reduced viability and induction of apoptosis in MG63 cells through down-regulation of caspase activation. Reference [3] stated that hydrogen peroxide caused strong oxidative stress and had shown to be a strong inducer of apoptosis in many cell types. Addition of 1- or 10-mM hydrogen peroxide induced reactive oxygen species (ROS) formation, oxidative DNA damage, dysfunction of mitochondrial membrane potentials, and early apoptotic changes in the human osteosarcoma cell line HS-Os-1. Therefore, [3], concluded that the formation of intracellular ROS is involved in hydrogen peroxide-induced apoptosis of osteosarcoma cells.

The superiority between hydrogen peroxide and liquid nitrogen based on the Huvos score requires further studies to validate the results of this study which further support the use of liquid nitrogen on the number of tumor cell deaths.

BMP-2 is a potent osteogenic factor that promotes the differentiation of mesenchymal stem cells into fibroblasts and chondroblasts. This protein consistently induces bone development. Reference [16], stated that BMP-2 is very highly expressed in osteosarcoma, and stated that BMP-2 can increase the growth of osteosarcoma cells. In addition, [17] stated that BMP-2 also exhibits an angiogenesis effect on tumors, by stimulating the expression of this important transcription factor for neo-angiogenesis of human dermal microvascular endothelial cells.

Based on the BMP-2 levels compared in this study, BMP-2 levels were found to be lower in the liquid nitrogen group compared to the group of core biopsy specimens of malignant bone tumors given hydrogen peroxide with a mean difference of 24.3. This was stated to be statistically significant based on

the independent t test (p value = 0.020).

A study by [18] examined BMP-2 expression in osteosarcoma cells from 75 patients given liquid nitrogen. The study results show that BMP-2 is overexpressed in osteosarcoma tissue. Meanwhile, interruption of BMP-2 expression with liquid nitrogen can inhibit the proliferation and invasion of osteosarcoma cells. BMP-2 is involved in the regulation of osteosarcoma cell proliferation and metastasis and can be used as a new molecular target marker for the diagnosis and treatment of osteosarcoma. This is consistent with the significance of the decrease in BMP-2 levels in the liquid nitrogen group found in this study.

In a study conducted by [16], which assessed BMP-2 levels in freezing with Liquid nitrogen found that the mean ± standard deviation in BMP-2 expression was 1.01 ± 0.07 in the control group, 0.73 ± 0. 07 in the irradiation group, and 0.88 ± 0.09 in the Liquid Nitrogen freezing group with p = 0.034. BMP-2 expression in bone tissue after radiation decreased significantly when compared to the control group [16]. In a study by [19], administration of liquid nitrogen had the advantage of anatomically compatible, cost-effective, and a simple and reliable reconstructive technique for reconstruction of bone defects after resection in patients with selective primary musculoskeletal sarcoma. Reference [20] stated that because it was widely available, the use of liquid nitrogen might be an option worth exploring in resource-limited environments when allografts and endoprostheses could not be obtained.

In the hydrogen peroxide group, ROS compounds caused oxidative damage and clinically reduced bone cell proliferation. This is in accordance with the study [21] which examined bone defects in mice. Administration of hydrogen peroxide caused a decrease in BMP-2 levels. Reference [22] argued that hydrogen peroxide could interfere with BMP levels and osteoinductivity of a graft. However, its role compared to liquid nitrogen still needs further research to confirm the results of this study.

Currently, studies were limited to in vitro studies. The disadvantage in vitro studies was that we could not predict the impact of complications from the usage of liquid nitrogen and hydrogen peroxide on healthy cells. As we know, there are still healthy cells around the tumor cells which are also affected by the treatment we are doing. Therefore, future studies may consider studying at the in vivo level using controls in healthy cells. In addition, future studies can also examine the best method and number of applications that can be carried out to produce the best output.

V. Conclusion

The number of tumor cell death in core biopsy specimens of malignant bone tumors with liquid nitrogen was higher than that with hydrogen peroxide. BMP-2 expression in core biopsy specimens of malignant bone tumors with liquid nitrogen was lower than that with hydrogen peroxide.

This study is the first study to compare cell death and BMP-2 expression in core biopsy specimens of malignant bone tumors with hydrogen peroxide and liquid nitrogen. Therefore, more in-depth further research is needed in the future regarding hydrogen peroxide and liquid nitrogen so it can be used as limb-salvage surgery and increase the survival rate of patients with malignant bone tumors.

Suggestions for further research ideas include the best way and number of applications that can be done to produce the best outcome. In addition to the normality of data distribution, the number of research samples can be increased to provide better statistical values.

Acknowledgment

We would like to thank I Wayan Suryanto Dusak as Head of Orthopaedics and Traumatology Department, Faculty of Medicine, Udayana University, Prof. dr. I.G.N.G. Ngoerah General Hospital for all the support regarding this study

Conflict of Interest

The author states that there is no conflict of interest related to the material discussed in the manuscript.

References

[1] Apley, & Solomon. System of orthopaedics and trauma. In Taylor & Francis Group. 2018.
[2] Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the surveillance, epidemiology, and end results program. *Cancer*. 2009.
[3] Ogawa Y, Takahashi T, Kobayashi T, Kariya S, Nishioka A, Mizobuchi H, et al. Mechanism of hydrogen peroxide-induced apoptosis of the human osteosarcoma cell line HS-Os-1. *International Journal of Molecular Medicine*. 2003; 12(4): 459-463.
[4] Rosen G, Marcove RC, Caparros B, Nirenberg A, Kosloff C, Huvos A. Primary osteogenic sarcoma. The rationale for preoperative chemotherapy and delayed surgery. *Cancer*. 197; 43(6): 2163-2177.
[5] Carreira AC, Lojudice FH, Halcsik E, Navarro RD, Sogayar MC, Granjeiro JM. Bone morphogenetic proteins: Facts, challenges, and future perspectives. *Journal of Dental Research*. 2014; 93(4): 335-45
[6] Kamal A. F., Rubiansyah P. Early clinical outcome of limb salvage surgery of osteosarcoma in the lower extremity using liquid nitrogen. *Bioscmed*. 2022; 6(6): 1875-83
[7] Feng C, Xu Z, Tang X, Cao H, Zhang G, Tan J. Estrogen-related receptor α: a significant regulator and promising target in bone homeostasis and bone metastasis. *Molecules*. 2022; 27(13): 3976.
[8] Sadykova LR, Ntekim AI, Muyangwa-Semenova M, Rutland CS, Jeyapalan JN, Blatt N, et al. Epidemiology and risk factors of osteosarcoma. *Cancer Investigation*. 2020; 38(5): 259-269.
[9] Van Mater D, Wagner L. Management of recurrent Ewing sarcoma: challenges and approaches. *Onco Targets Ther*. 2019; 12: 2279-2288.
[10] Dash A, Das T, Knapp FFR. Targeted radionuclide therapy of painful bone metastases: past developments, current status, recent advances and future directions. *Curr Med Chem*. 2020; 27(19): 3187-3249.
[11] Chen C, Garlich J, Vincent K, Brien E. Postoperative complications with cryotherapy in bone tumors. *J Bone Oncol*. 2017; 7: 13-17.
[12] Eka Wiratnaya, Ayu AAI, Yudhi Setiawan IGN, Wien IGNA, Suyasa IK, Kawiyana IKS, et al. Outcome of bone recycling using liquid nitrogen as bone reconstruction procedure in malignant and recurrent benign aggressive bone tumour of distal tibia: A report of four cases. *Journal of Orthopaedic Surgery*. 2017; 25(2): 2309499017713940
[13] Yamamoto N, Tsuchiya H, Tomita K. Effects of liquid nitrogen treatment on the proliferation of osteosarcoma and the biomechanical properties of normal bone. *Journal of Orthopaedic Science*. 2003; 8(3): 374-80.
[14] Nicholson NC, Ramp WK, Kneisl JS, Kaysinger KK. Hydrogen peroxide inhibits giant cell tumor and osteoblast metabolism in vitro. *Clinical Orthopaedics and Related Research*. 1998; 347: 250-260.
[15] Wang Y, Wang W, Qiu E. Protection of oxidative stress induced apoptosis in osteosarcoma cells by dihydromyricetin through down-regulation of caspase activation and up-regulation of BcL-2. *Saudi Journal of Biological Sciences*. 2017; 24(4): 837-842.
[16] Chen CM, Chen CF, Wang JY, Madda R, Tsai SW, Wu PK, et al. Bone morphogenetic protein activity preservation with extracorporeal irsarantion-and liquid nitrogen freezing-treated recycled autografts for

biological reconstruction in malignant bone tumor. *Cryobiology*. 2019; 89: 82-89.

[17] Raida M, Clement JH, Leek RD, Ameri K, Bicknell R, Niederwieser D, *et al*. Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. *Journal of Cancer Research and Clinical Oncology*. 2005; 131(11): 741-750.

[18] Chen X, Zhang Y. BMP-2 and miR-29c in osteosarcoma tissues on proliferation and invasion of osteosarcoma cells. *Oncology letters*. 2019; 17(6): 5389-5394.

[19] Garg SK, Aggarwal P, Virk J, Punia RPS, Dimri K, Jindal R. Limb salvage using liquid nitrogen-treated tumour-bearing autograft: a single institutional experience of 10 patients. *Indian Journal of Orthopaedics*. 2020; 54(2): 200-207.

[20] Hindiskere S, Doddarangappa S, Chinder PS. What are the challenges and complications of sterilizing autografts with liquid nitrogen for malignant bone tumors? A preliminary report. *Clinical Orthopaedics and Related Research*. 2020; 478(11): 2505.

[21] Han LJ, Ma F, Wang J, Pan H, Yang J, Zhao G, et al. Targeting endogenous hydrogen peroxide at bone defects promotes bone repair. *Advanced Functional Materials*. 2022; 32.

[22] DePaula CA, Truncale KG, Gertzman AA, Sunwoo MH, Dunn MG. Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties. *Cell and Tissue Banking*. 2005; 6(4): 287-298.