Catalase-expressing cell count and necrotic area in the chemotherapeutic response of osteosarcoma

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ABSTRACT

Introduction: Malignant cells have built-in mechanisms to protect themselves from the effects of chemotherapeutic agents. P53, via catalase, reduces reactive oxygen species (ROS) accumulation. P53 is mutated in almost all cancers, resulting in the accumulation of ROS and pro-tumorigenic signals. ROS can cause cell death through necrosis, apoptosis, or necroptosis. This study aimed to investigate the catalase-expressing cells and the necrotic area in osteosarcoma chemotherapeutic response.

Methods: This was observational analytic research using a retrospective design. The Huvos grades I, II, and III-IV, which indicate distinct degrees of tumor differentiation and aggressiveness, were compared. Formalin-fixed paraffin-embedded (FFPE) osteosarcoma tissue samples were histopathologically and immunohistochemically examined.

Results: The study of 27 samples revealed that 8 had a poor response, 14 had a moderate response, and 5 had a good response to chemotherapy. The percentage of necrotic area differed significantly (p=0.000) across treatment response groups. In contrast, there were no significant differences (p>0.05) in the number of catalase-expressing cells across treatment response groups.

Conclusion: ROS levels are increased in almost all cancer cells and play a crucial role in cell proliferation. In cancer, both oxidant and antioxidant systems may exert a greater influence on apoptotic pathways compared to the necrosis pathway.

Keywords: catalase, chemotherapy, Huvos grading, necrosis, osteosarcoma

INTRODUCTION

Osteosarcoma, a malignant form of bone cancer, poses significant risks to patients. The primary treatment options for osteosarcoma involve a combination of chemotherapy and surgical tumor removal. The specific treatment approach depends on factors such as tumor size, tumor location, and the individual patient's response to chemotherapy. While there have been advancements in osteosarcoma treatment, there remains a subset of patients who demonstrate a poor response to therapy and have lower survival rates.[1] Consequently, further study is imperative to identify the factors that impact patients' responses to chemotherapy.

A significant percentage of individuals had poor response or resistance to chemotherapy. From 1995 to 2008, data from Dr. Cipto Mangunkusumo General Hospital in Jakarta, Indonesia, indicated that 59.4% of patients were unresponsive or classified as Huvos grade I and II.[2] This conclusion is consistent with worldwide data, which show that a considerable number of patients had insufficient therapeutic responses. In a study conducted by Kim et al. (2017) in Korea, out of 56 analyzed patients, 26.8% exhibited a combined Huvos grade I and II or were unresponsive to treatment.[3] Another study by Letaief et al. (2020) in Tunisia explored prognostic factors and reported that out of 59 studied patients, 78% showed poor response to treatment with a median tumor necrosis rate of 70%.[4]

Nonetheless, the underlying mechanism behind the varied response to therapy in osteosarcoma remains uncertain, posing challenges in assessing treatment outcomes. While the Huvos grading system is used to assess response differences in osteosarcoma treatment, its mechanism is yet unknown. As a result, assessing the histological response to chemotherapy acts as a helpful prognostic marker for patients, allowing treatment efficacy to be determined. The goal of this study was to investigate the relationship between catalase-expressing cell count and necrotic area in osteosarcoma chemotherapeutic response.

MATERIALS AND METHODS

The number of catalase-expressing cells and the percentage of necrotic areas in the chemotherapeutic response of osteosarcoma were investigated using a retrospective observational analytic approach in this work. The study was conducted at the Laboratory of Anatomical Pathology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Hospital in Surabaya, Indonesia, for six months. All procedures of the study were approved by the Ethical Committee of Research at Dr. Soetomo General Hospital, Surabaya (Approval No. 0689/LOE/301.4.2/XI/2021).

The immunohistochemistry approach was used to compare Huvos grades I, II, and III-IV. The analysis utilized stored biological material in the form of formalin-fixed paraffin-embedded (FFPE) osteosarcoma tissue. The samples were obtained using a total sampling method from FFPE tissues of patients diagnosed with osteosarcoma, both with and without metastases, who had undergone surgery and received neoadjuvant chemotherapy. Over five years (2016-2020), the samples were gathered by total sampling from FFPE specimens of patients histopathologically identified at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital in Surabaya, Indonesia.

The inclusion criteria for the study consisted of male patients, aged equal to or below 20 years, with the presence of the lesion on long bones, who underwent resection or amputation, received neoadjuvant chemotherapy, and provided sufficient FFPE tissue from the osteosarcoma surgical material for histopathological and immunohistochemical examination. The exclusion criteria encompassed non-representative samples due to insufficient tissue for evaluation, specimen types other than resection or amputation, patients who did not get neoadjuvant chemotherapy, those who had radiation, and those who had "life-saving" amputations.

The evaluation of the necrotic areas involved both macroscopic and microscopic examination. The FFPE tissue samples were sectioned to a thickness of 3-5 μm and subjected to the hematoxylin and eosin staining method. Subsequently, the necrotic areas were observed under a light microscope at a magnification of 400x.

On the other hand, the number of catalase-expressing cells was assessed through immunohistochemistry. The tissue sections were incubated with the primary antibody of catalase monoclonal antibody (Cat. No. A11220, ABclonal, Woburn, MA, United States). The incubation was carried out in a humidified chamber at room temperature overnight. Following that, a labeled antibody was applied to each section. As a chromogen, diaminobenzidine was utilized (Cat. No. BDB2004, Biocare Medical, Pacheco, CA, United

States). After counterstaining, the sections were dehydrated with increasing concentrations of alcohol, mounted, and examined under a light microscope at a magnification of 400x.

A chemotherapy response assessment was conducted and categorized as poor, moderate, and good responses based on the Huvos grading method. Specifically, Huvos grade I indicated no necrosis or less than 10% necrosis, corresponding to a poor response. Huvos grade II represented 50-90% necrosis, indicating a moderate response. Huvos grade III reflected necrosis between 90-99%, indicating a good response. Finally, Huvos grade IV indicated 100% necrosis, signifying a complete response.

The collected data from this study were statistically analyzed to assess the differences and relationship between the number of catalase-expressing cells and the percentage of necrotic area in Huvos grades I, II, and III of osteosarcoma. Shapiro-Wilk test was conducted to test the normality of all variables. A one-way analysis of variance (ANOVA) test was performed to compare variables among all therapy responses, followed by Tukey's posthoc test. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 9 for Windows, Version 9.3.0, San Diego, CA, United States.

RESULTS

Table 1 presents the characteristics of the subject and osteosarcoma types. Out of the total 27 samples, 8 exhibited a poor response, 14 showed a moderate response, and 5 demonstrated a good response to chemotherapy. Fig. 1 shows that there were no significant differences ($p>0.05$) in the number of catalase-expressing cells between the groups. Fig. 2 illustrates the gross pathology inspection of osteosarcoma tissue, while Fig. 3 demonstrates the histological examination of the necrotic area. There were significant differences ($p=0.000$) in the percentage of necrotic areas between groups. The group that displayed a poor response to chemotherapy (Huvos I) exhibited a smaller area of necrosis and the presence of scattered viable malignant cells. In contrast, the group that exhibited a moderate response to chemotherapy (Huvos II) displayed a higher degree of necrosis and a smaller number of degenerated malignant cells. Lastly, the group that displayed a good response to chemotherapy (Huvos III) revealed a substantial area of necrosis with no viable malignant cells present.

Table 1. Subject characteristics and osteosarcoma types

Aspect	Total (n=27)	Poor Response (n=8)	Moderate Response (n=14)	Good Response (n=5)
Gender				
Male	27 (100%)	8 (29.6%)	14 (51.0%)	5 (19.4%)
Age (year)				
1-10	1 (3.7%)	0	1 (3.7%)	0
11-20	26 (96.3%)	8 (29.6%)	13 (48.1%)	5 (19.4%)
Osteosarcoma types				
• **Chondroblastic**	8 (30.0%)	2 (7.4%)	5 (19.4%)	1 (3.7%)
• **Osteoblastic**	7 (25.9%)	0	5 (19.4%)	2 (7.4%)
• **Fibroblastic**	3 (11.0%)	2 (7.4%)	1 (3.7%)	0
• **Giant cell-rich**	3 (11.0%)	1 (3.7%)	0	2 (7.4%)
• **Osteoblastic, fibroblastic**	3 (11.0%)	1 (3.7%)	2 (7.4%)	0
• **Osteoblastic, chondroblastic**	1 (3.7%)	0	1 (3.7%)	0
• **Osteoblastic, fibroblastic, chondroblastic**	2 (7.4%)	2 (7.4%)	0	0

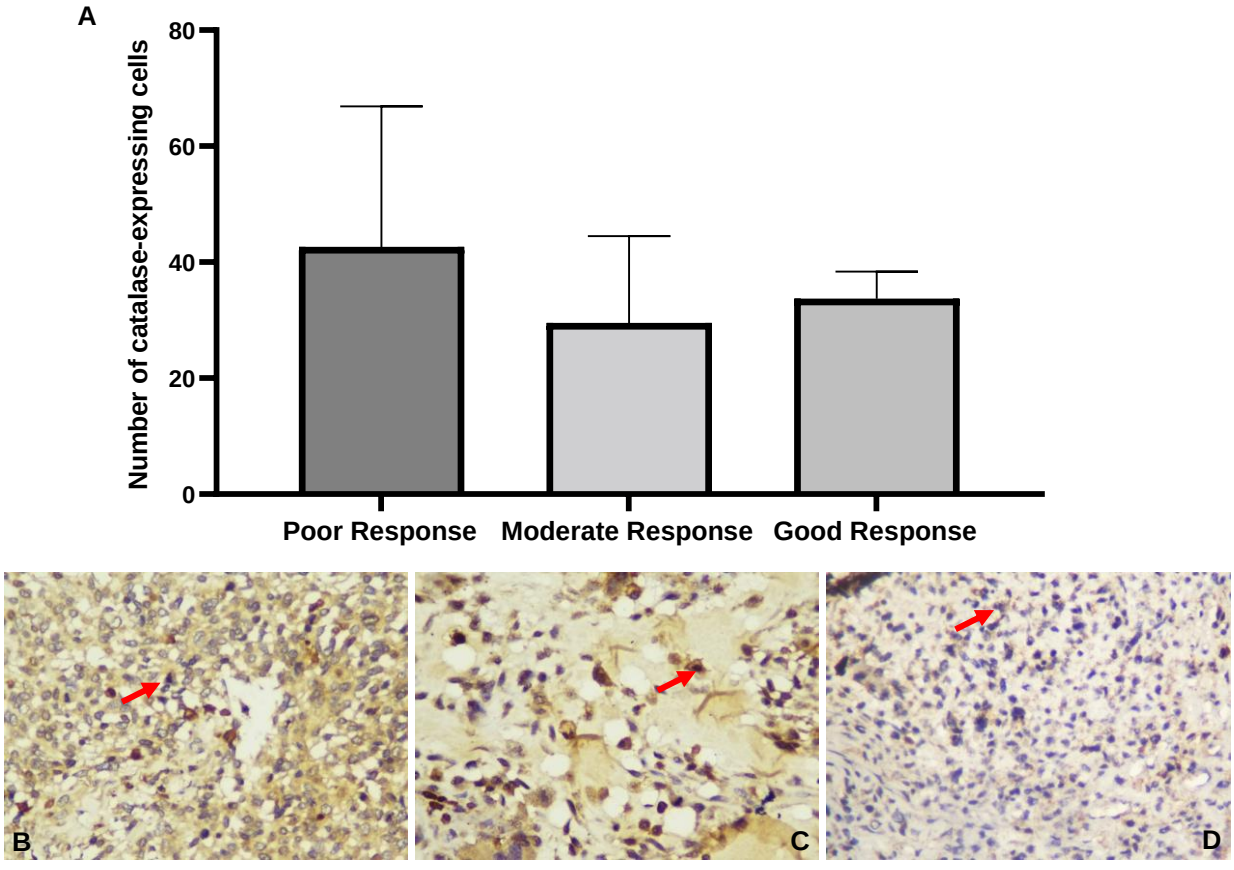

Fig. 1. Catalase-expressing cells.
The number of catalase-expressing cells was determined through immunohistochemistry, which involved counting under a light microscope at a magnification of 400x. There were no significant differences between the groups (A). The representative immunohistochemistry results show poor (B), moderate (C), and good responses to chemotherapy groups (D). Red arrows indicate positive staining cells.

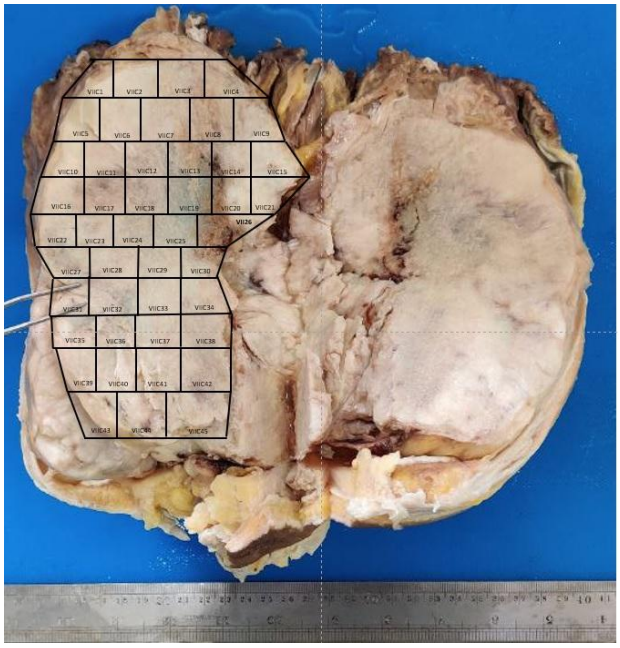

Fig. 2. Gross pathology inspection of osteosarcoma tissue.
The tissue was divided into squares, which were subsequently labeled. Histopathological sections were then prepared from each square of tissue. Courtesy of the Anatomical Pathology Department, Dr. Cipto Mangun Kusumo Hospital/Faculty of Medicine, Universitas Indonesia, 2023.

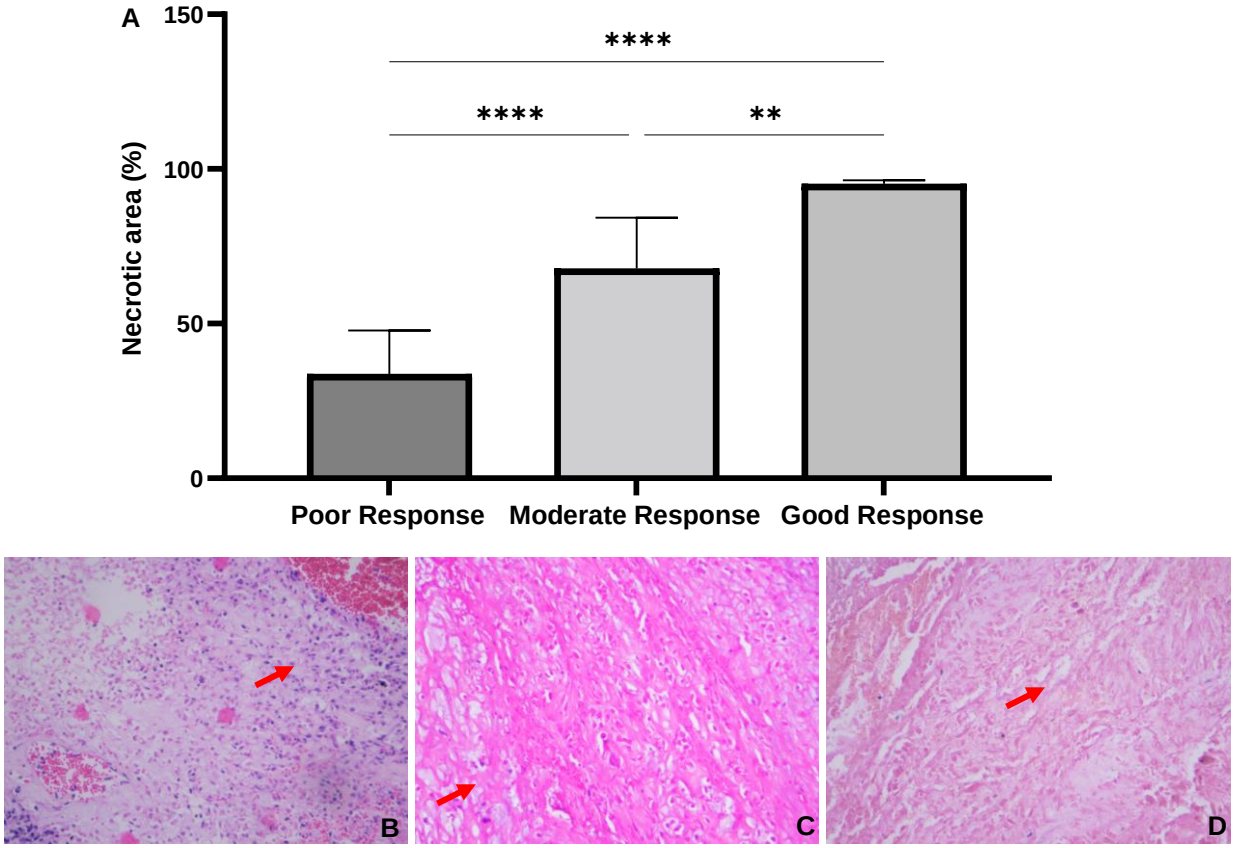

Fig. 3. Histological examination of necrotic area.
The percentage of the necrotic area was assessed by performing hematoxylin-eosin staining and examining the samples under a light microscope at a magnification of 400x. **p<0.01, ****p<0.0001 (A). The poor response to the chemotherapy group showed less necrosis and scattered viable

malignant cells (B). The moderate response to the chemotherapy group showed more necrosis and few degenerated malignant cells (C). The good response to the chemotherapy group showed a wide area of necrosis and an absence of viable malignant cells (D). Red arrows indicate necrosis.

DISCUSSION

Osteosarcoma, a rare bone tumor, has a low occurrence rate of 3.4 cases per million person-years worldwide.[5] It exhibits a bimodal age distribution, with one peak occurring during adolescence and another peak in older adulthood. According to data from Dr. Soetomo General Hospital, Surabaya, a total of 97 cases of osteosarcoma were recorded between 2016 and 2020, with 56.7% of the patients being male. The data also revealed that osteosarcoma is more common in individuals aged 11 to 20, accounting for 58.76% of all cases.

Osteosarcoma is a highly malignant, invasive, and progressing form of bone tumor with a high fatality rate.[6] Prior to the discovery of chemotherapy in 1970, the prognosis for patients with osteosarcoma was grim, with a survival rate of fewer than 20%. However, the introduction of surgical resection with sufficient margins and concomitant neoadjuvant treatment has boosted the survival probability to around 60-70%. Patients with localized osteosarcoma have a survival rate of 65%, whereas those with metastatic osteosarcoma have a survival rate of less than 20%.[7]

A multimodal treatment that includes neoadjuvant chemotherapy before surgery, followed by local surgical intervention and postoperative chemotherapy, has boosted the survival rate of osteosarcoma patients to 60-70% and extended their disease-free duration.[8] Current therapy techniques, however, have numerous drawbacks. Despite efforts such as dosage escalation, altering administration schedules, and using combination chemotherapy, survival rates have not improved since the advent of chemotherapy several decades ago. Furthermore, high-dose chemotherapy might have negative consequences. It is critical to find novel molecular markers that can define patient prognosis or predict treatment response in order to improve the prognosis of osteosarcoma patients. Additionally, researchers aspire to develop innovative and targeted therapeutic approaches, such as molecular-targeted therapy, specifically designed for osteosarcoma patients, to improve outcomes for individuals with a poor prognosis.[5,9] Thus, the objective of this study was to investigate the potential of the number of catalase-expressing cells and the percentage of the necrotic area as prognostic tools for evaluating chemotherapy response in patients with osteosarcoma.

The commonly utilized chemotherapy regimens for osteosarcoma include four chemotherapeutic agents: methotrexate with leucovorin rescue, doxorubicin, cisplatin, and ifosfamide.[5] It has been observed that trials utilizing three active agents yield superior results compared to those using only two regimens.[10] For first-line chemotherapy, the recommended options are cisplatin and doxorubicin (category 1), high-dose methotrexate, cisplatin, and doxorubicin (MAP) (category 1), and the combination of doxorubicin, cisplatin, ifosfamide, and high-dose methotrexate (MAPI).[11] In this particular study, patients received neoadjuvant chemotherapy following the guidelines provided by the National Comprehensive Cancer Network (NCCN), which included cisplatin and doxorubicin as the sole agents.

The extent of necrosis following neoadjuvant chemotherapy plays a vital role in determining the effectiveness of the treatment.[5] For most patients undergoing the current standard therapy of high-dose methotrexate with adriamycin and cisplatin (MAP), achieving a minimum of 90% necrosis is considered a favorable response to preoperative chemotherapy.[12,13,14] Cell death mechanisms, including programmed cell death such as apoptosis, autophagy, and necroptosis, as well as non-programmed cell death induced by chemotherapy, play significant roles in cancer treatment.[15] The previous study has indicated that conventional chemotherapeutic agents can increase the rate of necrosis in tumor cells by approximately 50%, irrespective of tumor volume and location.[16] The degree of chemotherapy-induced necrosis is strongly correlated with patient outcomes.[17] According to a

study conducted by Tsuda et al. (2020), the necrosis induced by MAP chemotherapy shows a positive association with Overall Survival (OS) or Event-Free Survival (EFS).[18]

In this study, significant differences were observed in the percentage of necrotic area among all response groups to chemotherapy. Furthermore, there were significant differences in the percentage of the necrotic area between the good response and poor response groups, as well as between the good response and moderate response groups and the moderate response and poor response groups. According to Li et al. (2011), treatment response varies across osteosarcoma patients and is reflected in histological results, notably the presence of necrotic cells.[19] Patients who respond well to treatment often have a better prognosis. Tsuda et al. (2020) discovered a favorable association between chemotherapy and satisfactory responses in osteosarcoma patients who had preoperative MAP treatment in a study involving 625 individuals under the age of 40.[17] The response to treatment is linked with specific histological features in resected tumor cells that indicate necrosis. The response influences the degree of necrosis in each cell, with a good response resulting in a higher degree of cell necrosis than a poor response.

Catalase-expressing cells have been identified as a possible predictor of treatment response in patients with osteosarcoma. Catalase, an antioxidant enzyme, plays a critical function in protecting cells from oxidative damage. It is mostly present in organs such as the liver, kidneys, and red blood cells. Catalase is typically found in the cytoplasm of cancer cells.[20]

It is conceivable that malignant cells possess mechanisms to protect themselves against the cytotoxic effects of chemotherapy. When osteosarcoma patients receive chemotherapy, the cellular damage caused triggers the generation of reactive oxygen species (ROS) within the osteosarcoma cells.[21] These ROS are enzymatically converted into water (H_2O) and oxygen (O_2) by catalase, which is produced by superoxide dismutase (SOD).[22] However, if the levels of SOD and catalase decrease, an excessive accumulation of ROS can lead to harm to cell components, increased damage to lipids, proteins, and DNA, and activation of programmed cell death. The high concentration of polyunsaturated fatty acids (PUFA) in the cell and organelle membranes renders them vulnerable to damage caused by ROS, a process known as lipid peroxidation.[23,24] This lipid peroxidation not only directly harms phospholipids but can also serve as a signal for programmed cell death.[25] In addition, osteosarcoma cells release a stress protein called heat shock protein-70 (Hsp-70) in response to counteract necrosis, which can increase the expression of scavenger proteins and Hsp-70 itself, thereby influencing osteosarcoma resistance to therapy.[26] Therefore, higher levels of catalase, SOD, and Hsp-70 have the potential to reduce the susceptibility of osteosarcoma cells to cell death and confer resistance to chemotherapy.[27]

The variable expression of catalase in tumor cells can influence the occurrence of cell necrosis.[28] Previous studies have also indicated the need for further study to better comprehend the potential of catalase in preventing oxidation and predicting chemotherapy response.[29] Similarly, a previous study conducted on patients with cervical cancer did not find a significant association between catalase levels and patient prognosis, indicating that catalase cannot be utilized as a marker for chemotherapy response in cancer patients.[30] The intriguing aspect of the dose dependence of oxidative stress is that high doses of ROS induce apoptosis or necrosis in cancer cells, while chronic exposure to low doses increases the survival and growth of cancer cells. The effect of ROS on cancer cell invasion is dose-dependent, as H_2O_2 triggers invasion only at doses ranging from 10 to 20 μM. ROS play a role in the survival, proliferation, resistance to apoptosis, neovascularization, invasion, extravasation, and metastasis of cancer cells (Griess, B. et al., 2017). These findings contribute to our understanding of the parameters that influence chemotherapy response in osteosarcoma. Notably, there were significant differences in necrosis levels among all treatment response groups, although they were not related to the antioxidant component. Antioxidant systems

may play a greater role in the apoptotic, neovascularization, invasion, and metastasis pathways than in the necrosis pathway.

CONCLUSION

These results emphasize the intricate nature of the factors that impact necrosis and treatment response in osteosarcoma. The study emphasizes the significance of conducting additional studies to enhance our understanding of the underlying mechanisms involved in necrosis and to identify alternative markers or factors that could potentially contribute to treatment response in this specific cancer type.

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AUTHOR CONTRIBUTION

S.M.: Conceptualization, data collection, and writing-draft; H.K., K.M., L.E.F.: Conceptualization, supervision, writing-review, and editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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