



The Co-treatment of Irradiated Tumor Cell Lysate Vaccine and Zinc Oxide Nanoparticles Motivates Antitumor Immunity

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THE USE of gamma radiation in vaccine production has proven to be one of the useful methods. Nanoparticle therapy, specifically the use of ZnO nanoparticles (ZnONPs), is a promising and innovative approach to treating a variety of diseases. The present study is designed to evaluate the effectiveness of irradiated tumor cell lysate vaccine and ZnONPs in treating solid Ehrlich ascites carcinoma tumors. The current study monitored the tumor size, MTT cell viability, and antioxidant markers (NO and SOD) in serum. Additionally, the levels of interleukins IL-10 and IL-6 were examined, as well as TGF- β , Caspase-3, and p53 expression in tumor tissues. The results demonstrated that the co-treatment of irradiated tumor cell lysate vaccine and ZnONPs had a potent effect in reducing tumor size, TGF- β , and P53 expression, and elevating IL-6 and IL-10 levels, and Caspase-3 activity. In conclusion, the combination of irradiated tumor cell lysate vaccine and ZnONPs shows great promise as a potential cancer treatment.

Keywords: Antioxidant, Cancer, Ionizing radiation, Irradiation, Tumor cell lysate vaccine, Zinc oxide nanoparticles

Introduction

Over the past few decades, several methods have been employed to treat cancer, a condition characterized by uncontrolled cell differentiation (Arneith, 2019). However, chemotherapy treatments, antimetabolites, biological agents, and natural products are not always effective since drug resistance and distinguishability of the cells can lead to systemic toxicity and debilitating adverse effects in normal body tissues (Guðmundsdóttir, 2023). Tumor cell-based vaccines derive tumor antigens from patient tumor cells (Sadeghi Najafabadi et al., 2022). Ex vivo genetic modification of the cells is followed by inactivation with high-dose IR or lysis. Tumor cell-based vaccines derive their tumor antigens from tumor cells obtained from patients (Hayes, 2021). Following ex vivo genetic modification, the cells are inactive via high-dose infrared (IR) (Remic et al., 2022). Irradiated whole-tumor cell vaccines are a type of radio-immunotherapy that can induce a wide range of tumor-associated

antigens (TAAs) (Al Saber et al., 2021). They are considered the best immunotherapy choice because the whole tumor cell presents the best source of immunizing antigens (Zhang et al., 2023). These vaccines have the potential to amplify the tumor-reactive T cells from the naive repertoire, reactivate existing tumor-specific T cells, and increase the extensiveness and variety of the tumor-reactive T cell response (Gray, 2021). This, in turn, minimizes immune escape. By stimulating T cell responses to these antigens, TAA in cancer vaccines can indirectly target tumors by releasing spontaneous tumor immunity (Saxena et al., 2021). The recognition of tumor cells by host immune cells has opened up new possibilities for eliminating cancer cells without the severe side effects of traditional cancer treatments (Chen et al., 2022). However, achieving a strong, tumor-specific immune response requires the use of adjuvants; various types of nanosystems have been utilized to protect and deliver vaccine components, showing a promise in achieving prolonged eradication of cancer

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cells (Yao et al., 2023). Zinc oxide nanoparticles (ZnONPs) are one of the most vital inorganic metal oxide nanoparticles utilized in different biomedical applications such as cancer therapy due to their remarkable properties and biocompatibility (Radwan et al., 2022). Zinc oxide nanoparticles have been found to exhibit cytotoxicity both in vitro and in vivo (Sengul & Asmatulu, 2020). These nanomaterials possess unique chemical properties (Czyżowska & Barbasz, 2022).

Furthermore, they have demonstrated a high degree of selectivity towards cancer cells, with the ability to surpass the therapeutic indices of traditional chemotherapeutic agents (Chandra et al., 2023). ZnO nanoparticles preferentially target rapidly dividing cancerous cells, leading to enhanced cytotoxicity through the generation of reactive oxygen species (ROS), which in turn, cause oxidative stress and eventual cell death, as described in the study by Hu et al. (2020). The present study focused on the effectiveness of the irradiated tumor cell lysate vaccine and ZnONPs in treating Ehrlich solid carcinoma by testing their role in apoptotic activation and host immunogenic activity enhancement.

Materials and Methods

Chemicals

Zinc oxide nanoparticles (ZnONPs) with a 20-50 nm diameter were purchased from Sigma-Aldrich Company Ltd. (purities 99.5 wt. %, surface areas 50 m²/g).

Animals

Fifty female Swiss albino mice (20-25 g) purchased from the National Center for Radiation Research and Technology, EAEE, Cairo, Egypt, were used in this study. Animals were maintained on a commercial standard pellet diet and tap water *ad libitum*. Care was consistent with the guidelines of Ethics by the Public Health Guide for the Care and Use of Laboratory Animals (National Research Center, 1996) by the proper care and use of laboratory animals approved by the animal care committee of the National Center for Radiation Research and Technology, Cairo, Egypt.

Tumor transplantation

Ehrlich Ascites Carcinoma (EAC) cell line was obtained from the National Cancer Institute (NCI), Cairo University. It was propagated in vivo as Ascites in female Swiss albino mice after

intraperitoneal (i.p) inoculation of 2.5×10^6 cells/mouse once a week for the time of the experiment (Salem et al., 2011). The solid Ehrlich carcinoma induction was performed by injecting 2.5×10^6 (7 days old) in the left thigh (Fahim et al., 1997).

Preparation of tumor cell lysate vaccine

According to Scchnurr et al. (2001), EAC cells were aspirated from the peritoneal cavity of EAC-bearing mice. The cell viability was measured using a hemocytometer and adjusted to $2.5 \times 10^6/\text{mm}^3$. EAC cells were irradiated with 8KGy γ -radiation (Salama & Hassan, 2014). The irradiated cells were incubated with 0.01% EDTA solution for 10 min, washed twice in PBS, and suspended at a density of $5 \times 10^6/\text{ml}$ in a serum-free medium. The cell suspensions were frozen at -80°C and disrupted by four freeze-thaw cycles (Scchnurr et al., 2001). Mice were injected weekly with 0.2 μl of cell lysate (supernatant) in the right thigh for three sequential weeks.

Experimental design

Fifty mice were divided into five groups, as follows:

1-Control group (C): mice received 0.2 mL saline. **2- EAC group (E):** mice were injected intramuscularly (i.m) with 0.2 ml of 2.5×10^5 ml/mouse viable EAC cells in the left thigh. **3-Ehrlich carcinoma/Lysate vaccine group (EV):** mice were injected i.m with 0.2 ml of tumor cell lysate vaccine in the right thigh one time /week for three weeks (Salama & Hassan, 2014); after two weeks, mice were injected with 0.2ml of 2.5×10^5 ml/mouse viable Ehrlich carcinoma cells in the left thigh **4- Ehrlich carcinoma/ ZnONPs (EZ):** mice developed tumors as a group (2) and after seven days; mice were injected intraperitoneally by ZnONPs (10 mg/kg, once daily for three weeks) (Dawood et al., 2019). **5-Ehrlich carcinoma/ Tumor cell lysate vaccine group / ZnONPs treated group (EVZ):** Mice developed tumors as in group (2) and were immunized as in group (3) and then treated with ZnONPs as in group (4). Two weeks later, after the EAC challenge, mice were sacrificed. Serum and tissues were collected for biochemical assays.

Tumor size mentoring

From the 6th day after Ehrlich carcinoma inoculation, tumor size was monitored using a Vernier caliper according to the following equation:

Tumor volume = 0.52 (length × width²)

where length is the greatest longitudinal diameter and width is the greatest transverse diameter (Jensen et al., 2008).

In vitro MTT assay

MTT cell proliferation and viability assay were applied according to Freimoster et al. (1999).

Biochemical assays

The present study measured oxidative stress markers (NO Nitric Oxide Assay Kit (Colorimetric) (ab65328) and SOD Superoxide Dismutase Activity Assay Kit (Colorimetric) (ab65354)) in the serum and estimated the immune mediators (IL-6 Rat IL-6 ELISA Kit (ab100772) and IL-10 Rat IL-10 ELISA Kit (ab214566)) in the tumor tissue. Caspase 3 levels were also detected in the tumor tissue using the ELISA kit from LSBio (Rat CASP3 / Caspase 3 (Sandwich ELISA) ELISA Kit - LS-F4138) as per the manufacturer's instructions.

Real-time quantitative polymerase chain reaction:

Tissue samples were processed with the RNeasy Mini Kit (Qiagen, Cat. No. 74104) to isolate total RNA under the protocol provided by the manufacturer. The first-strand complementary DNA (cDNA) was synthesized following the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (Qiagen, Cat. No. 205311) and 1 mg RNA as the template. Sequence Detection Software (PE Biosystems, CA) was utilized. 2 mL of cDNA, 900 nM of each primer (Table 1), and 2 mL of 2X SYBR Green PCR Master Mix (Qiagen, Cat. No. 204143) comprised the reaction mixture, which had a total volume of 25 mL. The PCR thermal cycling conditions contained 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C

for 20 s, with an initial step at 95 °C for 5 min. Utilizing the DDCT method, the relative expression of real-time reverse transcriptase PCR products was ascertained. This method calculates a relative expression to the housekeeping gene using the equation: fold induction $2^{-\Delta\Delta Ct}$. Where $\Delta\Delta Ct = [Ct \text{ gene of interest (unknown sample)} - Ct \text{ housekeeping gene (unknown sample)}] - [Ct \text{ gene of interest (calibrator sample)} - Ct \text{ housekeeping gene (calibrator sample)}]$ (Flamand et al., 2008).

Statistical analysis

Tests of significance were performed using the statistical package graph prism. All data are expressed as a mean of n= 6 values; SE and difference between means were considered significant at P < 0.05.

Results

Tumor size monitoring

The solid tumor volume was measured during the experiment. The solid tumor size was 131.37 mm³ on the 8th day after tumor injection. Tumor volume exceeded 349.40 mm³ on the 11th day; it continues to increase in size and reach 739.67 mm³ on the 13th day of tumor injection. However, mice treated with ZnONPs (EZ) showed an inhibition rate of 79.23%. Moreover, the tumor volume was diminished in mice immunized with the vaccine (EV) regarding the E group. The inhibition rate was 84.09% and the tumor volumes were 41.65, 56.56, and 75.35 mm³ on the 8th, 11th, and 13th day respectively. The group immunized with the vaccine and treated with ZnONPs (EVZ) showed a maximum percentage of inhibition by 89.85%, compared to the E group, also the tumor volume recorded at 31.82, 28.28, 48.19 mm³ on 8th, 11th, and 13th days respectively (Fig. 1).

TABLE 1. Primer sequences Used for RT-PCR. FW: Forward; Rv: Reverse

Gene	Primer sequence	Accession number
TGF-β	Fw TGATACGCCTGAGTGGCTGTCT Rv CACAAGAGCAGTGAGCGCTGAA	NM_011577
P53	Fw GTATTTACCCTCAAGATCC Rv TGGGCATCCTTAACTCTA	NM_001127233.1

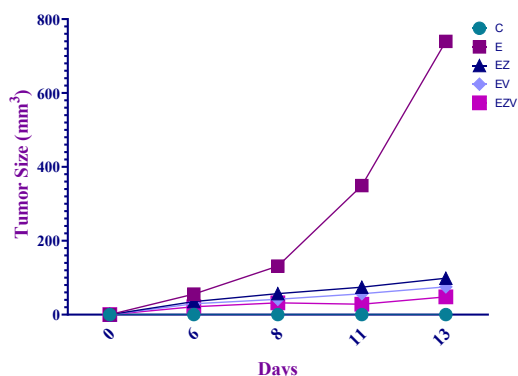


Fig. 1. Effect of irradiated tumor cell lysate vaccine and or ZnONPs on tumor size (mm³)

MTT viability assay

Figure 2 shows the results of the MTT viability test *in vitro*. EAC cells were incubated with different concentrations of irradiated tumor cell lysate vaccine (V) and ZnONPs. Viable EAC cells alone were considered as a positive control and recorded 100% viability. The effect of different concentrations of ZnONPs (50, 75, 100, and 150 μ l) on EAC cell viability showed a significant decline in EAC viability by increasing ZnONPs concentrations (56.4, 69.8, 89.7 and 91.28% respectively). Furthermore, EAC cells incubated with different concentrations of tumor cell lysate vaccine (100, 150, 200 and 250 μ l), revealed a dramatic cell inhibition of 95.3, 115.8, 131.6 and 136.2 respectively.

Biochemical examinations

Antioxidant activity

The antioxidant activities of the vaccine and ZnONPs were examined to determine their effectiveness in reducing nitric oxide levels and increasing SOD activity. Figure 3 demonstrates NO results in different groups; it showed that NO level was significantly raised in mice bearing tumors

(E) by 5.18-fold change compared to the normal group (C). However, treatment with ZnONPs resulted in a significant decrease in nitric oxide levels by 54.57% in mice with tumors (group EZ).. Similarly, vaccination with the irradiated tumor cell lysate vaccine (group EV) resulted in a significant reduction of nitric oxide levels by 24.03% compared to group E. Also, mice immunized with the vaccine and treated with ZnONPs showed a significant decrease in nitric oxide levels than group E.

In the present work, the tumor-developed mice (E) exhibited a significant decrease in SOD activity compared to the respective control 65.6%, whereas, the amelioration of SOD activity was clear in the group treated with ZnONPs. Similarly, vaccination with irradiated tumor cell lysate vaccine (EV) represented a significant increase in SOD activity (2.78 fold change) compared to the E group. Furthermore, the combination of both treatments documented a potent improvement compared to the E group (Fig. 4).

Immuno-mediator cytokines

In the current study, two immuno-mediator cytokines (IL-6 and IL-10) were examined that upregulate the immune system. The levels of both interleukins were significantly lower in the E group compared to the C group (39.3% and 54.4%) respectively. Conversely, treatment with ZnONPs (EZ) resulted in a marked increase in IL-6 and IL-10 levels (1.5 fold and 2.03 fold) when compared to the E group. Furthermore, in the vaccine group (EV), their levels showed a significant increase (1.67 and 2.23 fold in IL-6 and IL-10 respectively). Co-treatment of irradiated tumor cell lysate vaccine and ZnONPs led to a significant increase in both cytokines compared to the E group, as shown in Fig. 5.

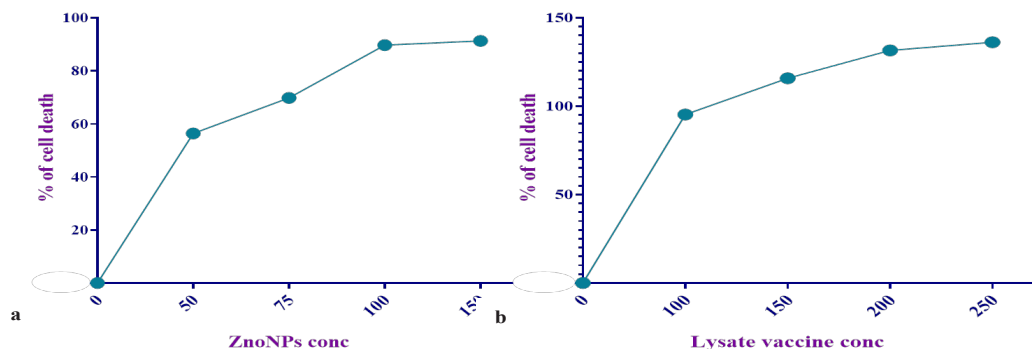


Fig. 2. Cell viability (%) of EAC incubated with different concentrations of (a) ZnONPs and (b) irradiated tumor cell lysate vaccine

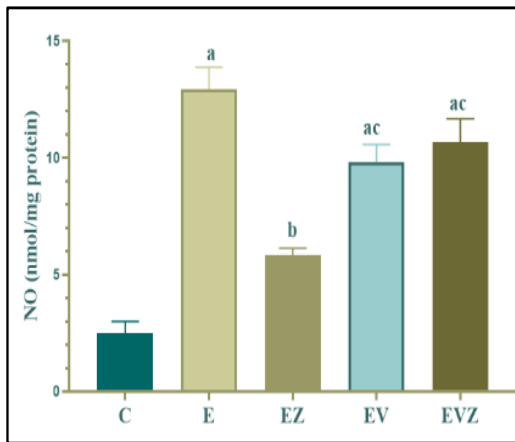


Fig. 3. NO level in different tested groups. Each value represents the mean \pm SE (n=6) [a: significant from control (P<0.05), b: significant from E (p<0.05), c: significant from EV (P<0.05); and d: significant from EZ (P<0.05)]

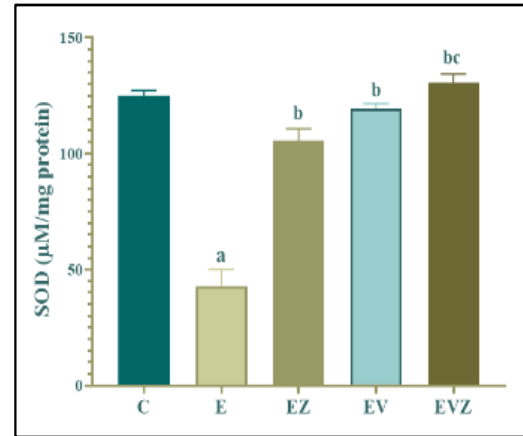


Fig. 4. SOD activity in different examined mice groups. Each value represents the mean \pm SE (n=6) [a: significant from control (P<0.05), b: significant from E (P<0.05), c: significant from EV (P<0.05); and d: significant from EZ (P<0.05)]

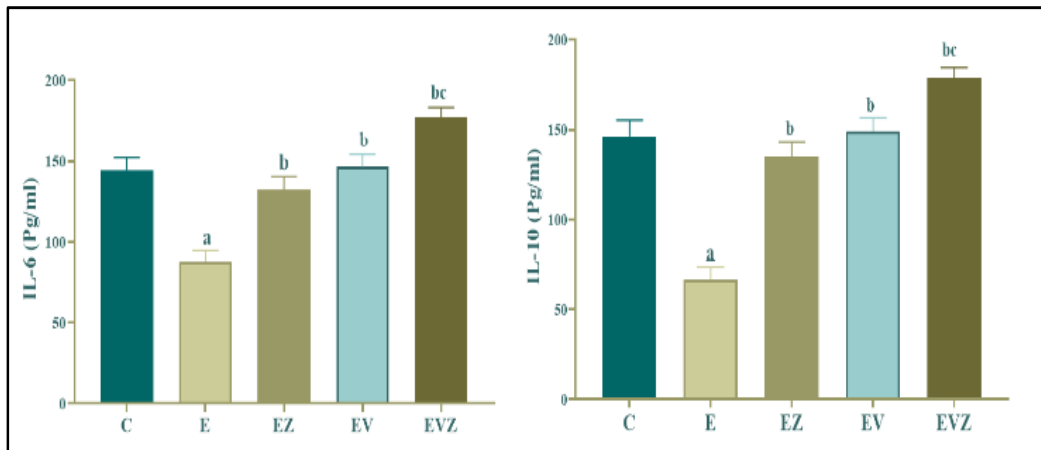


Fig. 5. a) IL-6 level in different estimated groups. b) IL-10 level in different estimated groups [Each value represents the mean \pm SE (n=6). a: significant from control (P<0.05), b: significant from E (P<0.05), c: significant from EV (p<0.05); and d: significant from EZ (P<0.05)]

The present study examined the expression of TGF β m-RNA (Fig. 6), which is a cytokine that can cause both pro- and anti-inflammatory responses in a cell, depending on the context. The control group (C) had an expression level of TGF- β at (3.40 \pm 0.45). However, the E group showed an essential over-expression of TGF- β , which reached (54.50 \pm 11.9) by 9.15 fold. On the other hand, treatment with ZnONPs (EZ), irradiated tumor cell lysate vaccine (EV), and the co-treatment group (EVZ) showed a reduction in TGF- β expression by 33.2%, 27.82 %, and 41.3% respectively, compared to the E group as shown in Fig. 6.

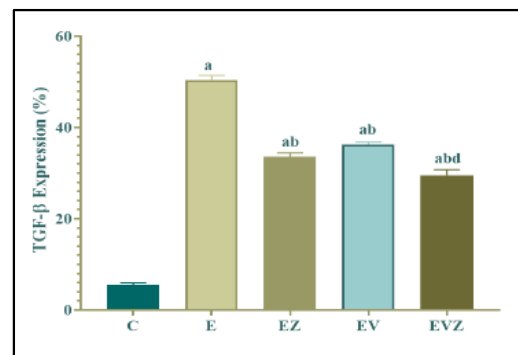


Fig. 6. TGF β m-RNA gene expression in different examined mice groups. Each value represents the mean \pm SE (n=6) [a: significant from

control ($P < 0.05$), b: significant from E ($P < 0.05$), c: significant from EV ($P < 0.05$); and d: significant from EZ ($P < 0.05$)

Apoptotic markers

The gene expression level of P₅₃-secreting cells was monitored in the tumor tissues. The mean concentration of P₅₃ in the secreting cells was (13.27) in the thigh muscle of the control group. However, a significant increase was identified in P₅₃ expression (74.10) in tumor tissue of the (E) group as compared to the (C) group. However, the administration of treatment of ZnONPs (EZ) and irradiated tumor cell lysate vaccine (EV), caused a decline in the expression of P53 (57.35% and 50.29%), respectively, compared to the (E) group (Fig 7a). Indeed, the best results were observed for the down-regulation of P₅₃ expression with the combined treatment (EVZ) (24.16%). Additionally, Figure (7b) exhibits the Caspase-3 activity. There was a potent elevation by 2.4 fold, in the mice that developed tumor (E) compared to the normal control. Besides, mice injected with ZnONPs (EZ) (1.79 fold change) showed a significant increase in Cas-3 compared to E group, while, the vaccinated mice (EV) display a non significant decrease regarding (E) (10.02%). More noticeable was the effect of the co-treatment group (EVZ) which showed a significant increase in Cas-3 (61.3%), compared to the (E) group as in Figure 7b.

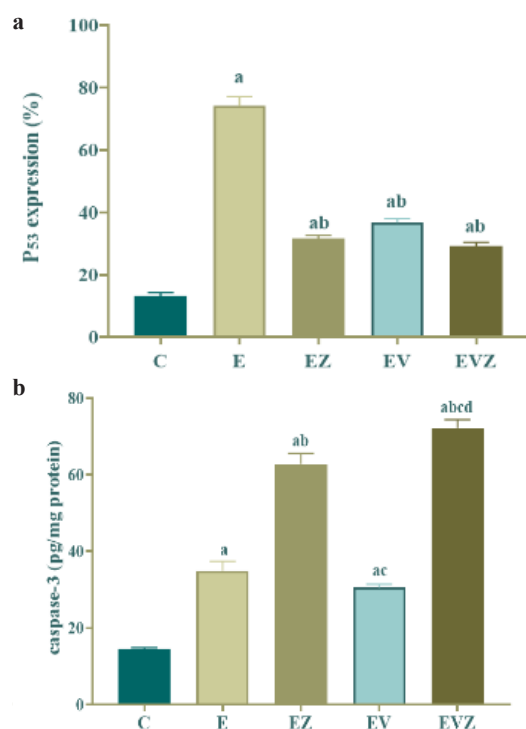


Fig. 7. P53 and Caspase-3 levels in different examined mice groups. Each value represents the

mean \pm SE (n=6) [a: significant from control ($P < 0.05$), b: significant from E ($P < 0.05$), c: significant from EV ($P < 0.05$); and d: significant from EZ ($P < 0.05$)]

Discussion

Conventional cancer treatment methods such as chemotherapy have been used for many years, but they are not always effective in eliminating cancer cells. The main reasons for this are drug resistance and the inability to distinguish between cancerous and healthy cells, which can cause harmful side effects. However, recent research has shown that the immune system can recognize cancer cells, which has led to the development of vaccine-based treatment protocols (Liu et al., 2022). In the current study, the tumor size was monitored, and the vaccine was found to significantly reduce tumor size either alone or in combination with zinc nanoparticles. Previous studies conducted by Hiserodt et al. (2007) and Luo et al. (2019) have shown that cancer vaccines can trigger the immune system to respond against tumors. This response can help slow down or eliminate the growth of tumor cells, even when they are located far away from the original tumor site. Essentially, the vaccine stimulates a systemic reaction against the tumor.

Reactive oxygen species (ROS) are produced in eukaryotic cells during aerobic metabolism. They have evolved into regulators of essential signaling pathways (Juan et al., 2021). Moderate ROS contribute to the regulation of cell proliferation and differentiation. Therefore, eukaryotic cells possess a complex scavenging system based on superoxide dismutases (SODs) located in the cytoplasm, mitochondria, and the extracellular matrix (Perillo et al., 2020). When cells experience oxidative stress due to increased ROS levels or decreased ROS scavenging, it can lead to cancer pathophysiology (Saleh, 2023). In the present work, oxidative stress-antioxidation imbalance led to SOD decrease and NO increase in the E group. However, the combination of the irradiated tumor cell vaccine and ZnONPs demonstrated an increase in SOD levels and a decrease in NO levels in the EVZ group when compared to the E group. This proves a synergistic cytotoxic effect of both treatments against tumors. These findings are in harmony with a previous study that found that oral administration of ZnONPs (at a dosage of 300-500 mg/kg) inhibited the growth rate of Ehrlich solid carcinoma (El-Shorbagy et al., 2019). Furthermore, Dawood et al.

documented that ZnONPs have selective killing properties due to the generation of ROS (Dawood et al., 2019). Moreover, Khan et al. (2020) have previously documented that NO has both pro- and anti-tumor effects in cancer biology. It plays an important role by altering the cellular response to stressors such as DNA damage, oncogene activation, and metabolic changes (Chavda et al., 2022). Additionally, it can deregulate DNA repair enzymes and tumor suppressor genes, while modulating apoptotic and metastatic processes (• Ramírez-Patiño et al., 2022).

IL-6 is a type of cytokine that has multiple effects on immune response and inflammation (Hirano, 2021). IL10 plays a crucial role in the differentiation of regulatory T cells, which is important for controlling immune responses and developing immune tolerance (Grover et al., 2021). The present study revealed a significant decrease in serum IL-10 and IL-6 levels in the E group compared to the C group. This finding supports previous research indicating that mice lacking IL-10 or IL-10 receptors have a higher likelihood of developing tumors (Mirlekar, 2022). Additionally, the administration of exogenous IL-10 has been shown to enhance antitumor immunity in mice (Salkeni & Naing, 2023). Furthermore, the study found that the vaccine and nano zinc combination had a synergistic antitumor effect. This was demonstrated by higher levels of IL-6 and IL-10 secretion in vaccinated animals, either alone (EV) or in combination with nano zinc (EVZ), compared to the E group. This is in line with Chen et al. in their study conducted in (2021), the combination of vaccination and other factors can lead to an increase in the expression of surface costimulatory molecules and cytokine secretions, which in turn, enhances cytotoxicity against tumors mediated by cytotoxic T lymphocytes (CTL) (Chen et al., 2022). The use of a booster vaccination with tumor cell lysate can effectively activate the antigen by dendritic cells (DCs), which then stimulate tumor-specific memory CD8+ T cells through the cross-presentation of the tumor antigen. Additionally, these DCs secrete high levels of IL-6 and IL-12, resulting in the induction of effective antitumor responses. The study conducted by Shui et al. (2023) supports this claim.

The transforming growth factor beta (TGF- β) is a highly potent cytokine that has pleiotropic effects on cells and regulates their behavior (Xue

et al., 2020). In tumors, TGF- β can act as either a proto-oncogene or a tumor suppressor, depending on the cellular context and tumor stage (Baba et al., 2022). For example, in the current study, the group that developed tumor (E) showed a significant elevation regarding to normal mice(C), which means that TGF- β acts as a proto-oncogene, as supported by Huppert and his colleagues, who stated that TGF- β over-expression is linked to worse outcomes and has been shown to engage with breast cancer metastasis (Huppert et al., 2022). On the other hand, the treatment of the vaccine showed a potent amelioration in the TGF- β mRNA expression both alone and in combination with ZnONPs.

Several studies have suggested that reducing the levels of mutant p53 can significantly increase apoptosis (Guo et al., 2020). In the present study, our results indicated that rats exposed to E had a potent increase in P53 gene expression, while the EV group showed a decline in the same gene expression. Furthermore, the current findings revealed that the combination of zinc nanoparticles and irradiated vaccine resulted in a significant decrease in the gene expression of mutated P53 compared to the E group. This is consistent with previous research that has linked lower levels of mutant p53 to an increase in apoptosis, suggesting that these cells may depend on mutant p53 for their survival (Roszkowska et al., 2022). Zinc oxide nanoparticles (ZnONPs) have been shown to induce the wild type of P53 and decrease the mutant type by enhancing apoptosis (Singh et al., 2020). The obtained results align with a previous research that has documented ZnONPs' ability to activate Bax and P53 as a pro-apoptotic step (Jain & Tailang, 2023). Chen et al. supported that the p53 gene is a significant antioxidant regulator in ZnONPs-induced cell death. Additionally, a previous research has shown that ZnO NPs can inhibit the proliferation of mast cells by regulating MDM2 and p53 protein levels (Chen et al., 2022). Caspase 3 activation initiates the progress of the upstream apoptotic process, which is the vital step in apoptosis (McComb et al., 2019). The present study showed a significant elevation of caspase-3 activity after administration of irradiated tumor cell lysate vaccine with ZnONPs co-treatment. Furthermore, Wang et al. demonstrated that the caspase cascade was activated by ZnO-NPs (Wang et al., 2020). Chen et al. documented that despite the variability of vaccines, irradiated tumor cell lysate vaccine causes caspase 3 activation

(Chen et al., 2023). Notably, the elevation in the caspase cascade may be due to the increase in NO produced by iNOS that directly stimulates GC to AT mutations in p53 which may contribute to the loss of its repressor activity, and inhibit Caspase 3 activity, providing an efficient means to block apoptosis (He et al., 2022). This study is just the first step towards finding an effective combination for cancer treatment. Further research on different types of carcinoma is required to achieve this goal.

Conclusion

The present study proposes vaccine irradiation-based immunotherapy as a potential approach for treating tumors. The irradiated vaccine showed a significant antitumor activity, making it an effective therapeutic option. Additionally, immunization with the cell lysate vaccine acts as an immunomodulator and is particularly effective when combined with ZnONPs. These findings suggest using irradiated tumor cells as a vaccine adjuvant for other whole tumor cells.

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