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Research Paper

Impact of Modified ZnO Nanoparticles on Immune Response in Mouse Lung Cancer Model

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ABSTRACT

Background: Lung cancer, which is characterized by the presence of malignant tumors, offers a potential avenue for treatment through the use of nanomedicines. Previous in vitro studies have shown promising effects of modified zinc oxide nanoparticles on lung cancer cell lines. Accordingly, this study investigates the impact of this nanodrug on the immune response in a mouse model of lung cancer.

Materials and Methods: In this study, a mouse model of lung cancer was utilized. Various aspects, including tumor size, infiltration of $CD8^+$ cells and the survival rate of the mice, were carefully examined. The obtained results were subsequently analyzed using the GraphPrism software, version 9.

Results: Mice treated with the nanodrug exhibited a reduction in tumor size. Additionally, there was an increase in the number of $CD8^+$ cells infiltrating the tissue. Furthermore, the administration of the nanodrug led to improved survival rates among the mice.

Conclusion: The use of this nanodrug has shown significant efficacy in inhibiting tumor growth. Moreover, it has demonstrated potential in enhancing $CD8^+$ cell infiltration, thereby strengthening the immune response and suppressing tumor progression. Ultimately, this nanodrug improves the survival of mice receiving treatment.

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Introduction

Cancer, a group of diseases characterized by abnormal cell growth with the potential to invade or spread to other parts of the body, presents a significant challenge in the field of medicine. Human beings can develop over 100 different types of cancer, with extent of metastasis [1]. Lung cancer, specifically lung carcinoma, is a malignant tumor that arises from uncontrolled cell growth in lung tissues and primarily originates from epithelial cells [2, 3]. The treatment approach for lung cancer depends on various factors, including the specific type of cancer cells, the extent of metastasis and the overall functional status of the individual. Common treatment modalities include palliative care, surgery, chemotherapy, radiotherapy, and immunotherapy [4, 5]. Notably, there is increasing emphasis on the development of targeted and personalized therapies for advanced lung cancer. Despite the ongoing efforts of cancer researchers, there is currently no definitive cure for this disease, and research continues to explore potential solutions. Furthermore, conventional cancer treatments often have significant side effects. Many anticancer drugs are designed to not only eradicate cancer cells but also hinder the growth and proliferation of healthy cells, which can result in adverse effects on the host organism. In light of these challenges, scientists are actively pursuing treatment methods that minimize the side effects and exhibit a high level of specificity in targeting cancerous cells. One promising approach is the utilization of nano-drugs, which have the potential to deliver therapeutic agents with enhanced precision and selectivity [6-8].

Nanomedicine, a branch of nanotechnology, offers immense potential in the fields of medicine and research by providing powerful and widely applicable tools [9]. One of the key advantages of utilizing nanotechnology in medicine is the ability to modify nanoparticles such that they can effectively differentiate between normal and damaged tissues [10]. For example, cancer cells often exhibit distinct molecular markers that differentiate them from healthy tissues [10]. It is possible to selectively target and enter cancer cells by designing nanoparticles that can specifically bind to these unique molecules [11]. This approach holds significant promise, particularly in resource-constrained regions, where patients may not have access to expensive cancer treatments. Compared to conventional methods, such as chemotherapy, radiotherapy, and immunotherapy, the cost of utilizing nanotechnology for drug research and development is considerably lower, making it a cost-effective solution for society [12]. One example of a nanomedicine with anti-cancer properties is zinc oxide (ZnO) nanoparticles [13]. However, these nanoparticles alone are not suitable for medicinal use because of their limited fluidity and non-specific toxicity [14-16]. To overcome these limitations, modified ZnO nanoparticles have been developed using nanotechnology [17]. In this formulation, ZnO nanoparticles were synthesized in the form of nanoparticles along with nanocomposites possessing reduction properties. Subsequently, a surfactant and ethoxylated wetting agent were added to the polyethylene glycol polymer nanoparticles. Finally, the nanoparticles were modified with a hydrophilic sorbitan emulsifier to ensure dissolution at a pH of 7-8. Currently, this drug is undergoing additional in vitro and in vivo studies to evaluate its efficacy further [17].

The immune system's responses play a crucial role in cancer [18]. Analyzing these responses can aid in elucidating the mechanism of action of the drug and in forecasting the prospective trajectory of the disease, encompassing re-expansion, recurrence, or full clearance [19]. Among various immune responses, cellular immune responses are particularly significant in cancer research. $CD8^+$ and cytotoxic cells, which are responsible for eliminating cancer cells and developing memory against specific types of cancer, serve as valuable indicators [20]. In such studies, it is more informative to examine the lymphocytes present in the cancer microenvironment, as they have more specific functions.

Accordingly, this study assesses the impact of the aforementioned nanodrug on a mouse model of lung cancer. Key parameters, including $CD8^+$ cell infiltration, tumor size, and survival were measured.

Materials and Methods

Study design

In this study, four groups, each consisting of five mice, were included. For the 15 and 30-day drug groups, a modified ZnO nano-drug was administered orally at a dose of 30 ng/mouse every other day, along with a regular diet. The 15- and 30-day drug solvent groups received 100 µL of the drug solvent orally in a feeding tube and diet every other day. Groups of 15 mice were euthanized on the 15^{th} day of the study following ethical guidelines. Groups of 30 mice were observed for survival analysis until the 30^{th} day and any surviving mice were euthanized for further analysis in compliance with the ethical guidelines.

Induction of lung cancer model in C57BL/6 mice

To induce the mouse model, the first step involved obtaining the LL2 cell line from the Pasteur Institute of Iran. The cells were then cultured and injected into mice. The cells were cultivated in an incubator with 5% CO_2 and 92% humidity, using Dulbecco's modified eagle medium culture medium (Gibco, Cat# 11-965-092) supplemented with 10% fetal bovine serum (DNAbiotech, Cat# DB9723-100ml, I.R. Iran) and penicillin-streptomycin (Gibco, Cat# 15140). Initially, live LL2 cells were counted using a hemocytometer slide and a trypan blue dye (DNAbiotech, I.R. Iran). At least 2.5×10^6 cells were suspended in 500 µL of 5% matrigel-phosphate buffered saline (PBS) (Corning, Cat# 354234), and 0.01 M ethylenediaminetetraacetic acid (Titriplex, Cat# 108417). Subsequently, five experimental mice were chosen and each was administered with 100 µL of cell suspension containing an estimated 5×10^5 LL2 cells in the flank region. After 15 days, tumor growth was observed in the flank region and the tumor volume reached the desired size for transplantation in 25 mice by the 50th day. Three days before transplantation, cyclosporine was administered to mice at a dose of 15 mg/kg. On the day of transplantation, the mice were initially anesthetized using ketamine-xylazine (ketamine 100 mg/kg and xylazine 10 mg/kg), and subsequently, a piece of tumor tissue measuring 2-3 mm^3 from the pilot mice was implanted in their flank region. After 15 days, the tumors of 20 mice were clinically confirmed and subjected to further investigation.

Immunohistochemical staining

To investigate the infiltration of $CD8^+$ cells into the tumor area, we performed immunohistochemistry on tissue samples obtained from the tumor region. A veterinary pathologist confirmed the cancerous nature of these tissue samples and subsequently prepared the slides. Additional tissue slides from the same tissues were also obtained for immunohistochemical evaluation of CD8 markers. The paraffin sections were then subjected to softening in an oven at temperatures ranging from 57˚C to 60˚C for 40 min. The slides were deparaffinized by immersion in xylene (Neutron, Cat# 1.1570, Iran) three times for 30 min each. Subsequently, the slides were rehydrated by immersing twice in graded ethanol (Merck, Cat# 1.00986) (100%, 90%, 70% and water) for 3 min each time. The intrinsic peroxidase activity of the tissue was neutralized by immersing the slides in a methanol (Merck, Cat# 106007) solution containing 30% hydrogen peroxide (DNAbiotech, Cat# DB9651-25 mL, I.R. Iran) for 20 min (20 mL of 30% hydrogen peroxide+80 mL methanol). The slides were washed thrice with PBS and tap water. Thereafter, the slides were placed in preheated sodium citrate buffer (AP-RAD, Cat# APSSCK1, Iran) and incubated at temperatures ranging from 95˚C to 100˚C for 15–30 min. After cooling, the slides were washed thrice with PBS and tap water. The slides were blocked with the appropriate blocking buffer for 2 h at room temperature. After removing the blocking buffer, the slides were incubated overnight with a suitably diluted primary antibody (Abcam, Cat# ab217344) in 0.1% PBS-bovine serum albumin. The slides were then incubated with biotinylated secondary antibody (Abcam, Cat# ab64256) diluted at a ratio of 1:200 in 0.1% PBS-bovine serum albumin for 1 h at room temperature. The slides were then treated with the ABC solution (Thermo Scientific, Cat# 32020) for 1 h at room temperature. To stain the antigens in the tissue, 3,3′-diaminobenzidine solution (Abcam, Cat# ab64238) was applied to the slides for ≤3 min. Light nuclear staining was performed and the slides were counterstained with hematoxylin (Sigma-Aldrich, Cat# H3136) for 4–5 min. Excess hematoxylin was removed by briefly dipping the slides in acidic alcohol. The reblued slides were immersed in ammonia water, followed by immersion in graded ethanol (70%, 85% and 100%) twice for 3 min each. Finally, the slides were dehydrated by immersion in xylene for 30 min. The slide was mounted using Permount Mounting Media (Permount, Cat# FSCSP15) and prepared for observation under a microscope.

Data analysis

Statistical analysis of the data was conducted using the GraphPad Prism software, version 9.5.1. The analytical approach employed included descriptive statistics, including measures, such as mean and standard deviation, in addition to inferential statistics used to compare groups. The statistical tests employed for general comparisons included a two-way repeated measure analysis of variance, and for survival analysis, the Kaplan-Meier method was utilized. Furthermore, the normality of the data was assessed using the Shapiro-Wilk test. Additionally, Grubbs' test was employed to confirm outlier data and post hoc analysis after analysis of variance was performed using the Tukey honestly significant difference test, among others.

Result

Tumor growth reduced by nanodrug

To assess the efficacy of the nanodrug, an investigation was conducted on the size of tumors in a group of mice. The tumor size of 15 groups of mice was measured using a digital caliper at the beginning of the study and then

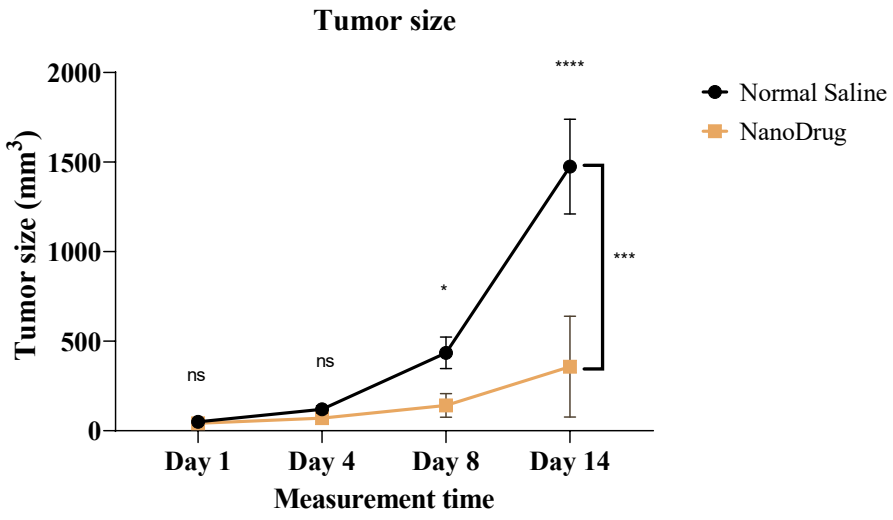

Figure 1. The trend graph depicting the size of tumor growth revealing considerable findings

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Notes: Over the course of 15 days, the tumor size changes in mice belonging to 15 different groups were observed and analyzed. Notably, the mice in the drug solvent group exhibited a significant increase in tumor growth compared to other groups. Conversely, the mice receiving the nanodrug demonstrated a relatively slower tumor growth process. To evaluate the statistical significance of these observations, a 2-way repeated measure analysis of variance was conducted. The results indicated a significant difference between the groups (P=0.0002), suggesting that the type of drug administered had an impact on tumor growth. Additionally, there was a significant difference observed over time (P=0.0001) and between different groups (P=0.0084). Given the significance of the analysis of variance results, a comparison between the different groups was considered necessary. To accomplish this, the Bonferroni multiple comparisons test, as an accepted post hoc test for this type of data, was employed.

recorded every four days until the end of the study. Subsequently, the average tumor size for each group was calculated and subjected to statistical analysis. As depicted in Figure 1, the mice receiving the nanodrug exhibited less tumor growth over the course of the 15-day study compared to those receiving the drug solvent. This pattern of tumor growth was also examined in a 30-day survival analysis, which exhibited similar results. Specifically, the mice in the nanodrug group displayed reduced tumor growth compared to the drug solvent group.

The infiltration of $CD8^+$ cells into the tumor tissue exhibiting an augmentation

In this study, the objective was to investigate the impact of a modified ZnO nanodrug on the infiltration of $CD8^+$ cells in tumors through immunohistochemical evaluation. Following the immunohistochemical test, five random sections of the slides were photographed, and the cells were counted for subsequent statistical analysis. The results, as depicted in Figure 2, demonstrated that in the group receiving the drug solvent on day 15, the infiltration of $CD8^+$ cells was observed to be rare when examining various tissue areas. However, one mouse in this group exhibited a remarkably high infiltration of $CD8^+$ cells. Conversely, in the group receiving the nanodrug on day 15, there was widespread and diffuse infiltration of $CD8^+$ cells in the tissue areas. In these regions, the color intensity was more pronounced compared to the cisplatin group, as illustrated in Figure 2. Meanwhile, statistical analysis revealed a significant difference in the average number of infiltrating $CD8^+$ cells between the groups receiving the nanodrug and those receiving the drug solvent.

Nanodrug resulting in a significant improvement in the overall survival rates

From the beginning of the investigation, mice belonging to the groups of 30-day were subjected to meticulous examination by a proficient veterinarian, as these groups were deemed suitable for survival analysis. The mice underwent daily clinical assessments, and their death was promptly documented. The findings of the survival analysis are in concordance with those shown in Figure 3. Mice administered the drug solvent exhibited a shorter lifespan compared to the other group. Notably, all mice in the group administered the drug solvent survived until the 15^{th} day of the study. Interestingly, two mice from the nanodrug group demonstrated prolonged survival until day 30 without any noteworthy clinical complications. However, they were subsequently sacrificed for further analysis.

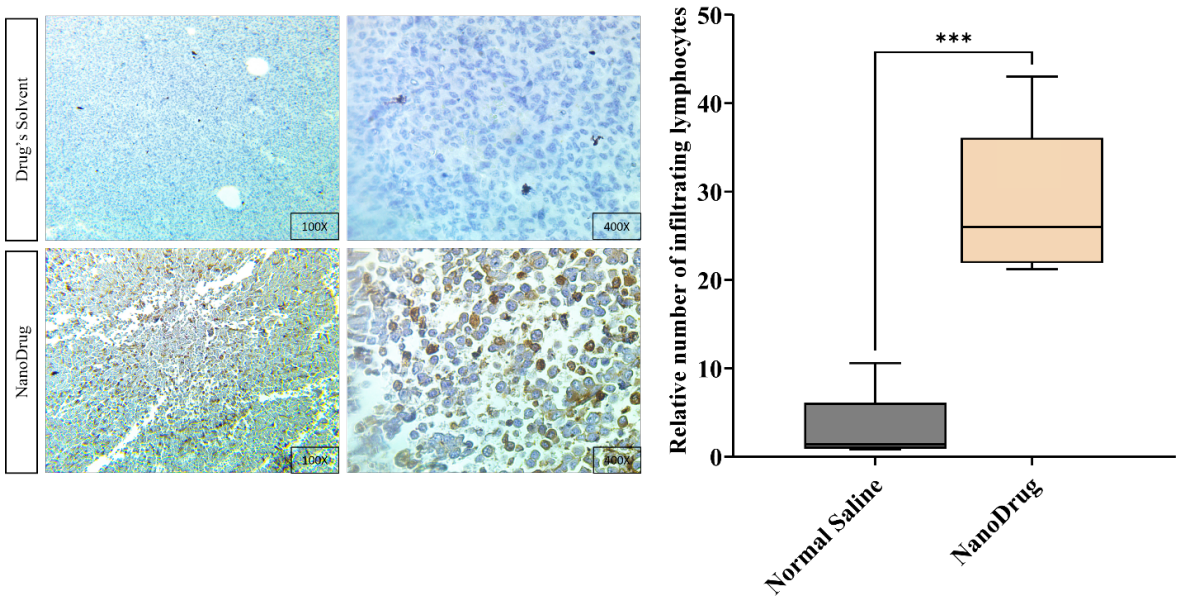

Figure 2. The evaluation of CD8 cell infiltration into tumor tissue

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Notes: An immunohistochemical test was performed on tumor tissue samples, and an image of one mouse from both the nanodrug and drug solvent groups was selected as a representative example. The image was captured at two magnifications, 100 and 400. In the image, cell nuclei were stained blue, while the CD8 marker was stained brown. Notably, the presence of $CD8^+$ cells, indicating lymphocyte infiltration into the tumor tissue, was observed in mice treated with nanomedicine. Subsequently, the infiltrated cells were counted, and a corresponding statistical analysis was conducted. The normality of the data was assessed using the Shapiro-Wilk test, followed by a t-test to compare the two groups. The obtained P was P=0.0004, indicating a significant difference in the average number of infiltrated cells between the two groups.

Discussion

This study assessed the impact of modified ZnO nanodrug on the immune system. As $CD8^+$ lymphocytes are crucial immune cells in the tumor microenvironment, their presence was examined to determine whether stimulation of the immune system by ZnO nanoparticles leads to an appropriate immune response. Additionally, a survival analysis was conducted. Several other factors could have been explored in this study but were not due to constraints, such as time and financial resources. The aforementioned factors are the most significant factors discussed.

The size of a tumor, which serves as an indicator of the growth of cancer cells, is a crucial factor in determining the efficacy of anticancer drugs [21]. If a particular drug under investigation demonstrates effectiveness, it should exhibit the ability to reduce tumor size in comparison to control groups [22]. This reduction in tumor size can be attributed to the drug's ability to impede the growth and division of cancer cells or its capacity to eliminate as many cancer cells as possible within the tumor region [23]. As tumors increase in size, they become progressively more challenging to treat. Consequently, this study explored the relationship between tumor size and the effectiveness of anticancer nanomedicine. Based on the findings presented in Figure 1, mice administered with the nanodrug exhibited smaller tumor sizes compared to those receiving the drug solvent. Similar results were observed among mice in the 30-day group. Therefore, the modified ZnO nanodrug contributes to a decrease in tumor growth.

Recent studies have demonstrated that the administration of anticancer drugs can effectively enhance the infiltration of $CD8^+$ T cells into the tumor microenvironment, ultimately resulting in improved clinical outcomes for cancer patients [24-26]. One specific drug that exhibits this capability is pembrolizumab, an immune checkpoint blockade that specifically targets programmed cell death protein receptor 1 on T-cells. Preclinical investigations have indicated that pembrolizumab promotes the infiltration of $CD8^+$ T cells into the tumor microenvironment, thereby leading to enhanced antitumor activity [27]. Similarly, another drug known as ipilimumab, which functions as an immune checkpoint blockade targeting cytotoxic T lymphocyte-associated antigen 4, has also

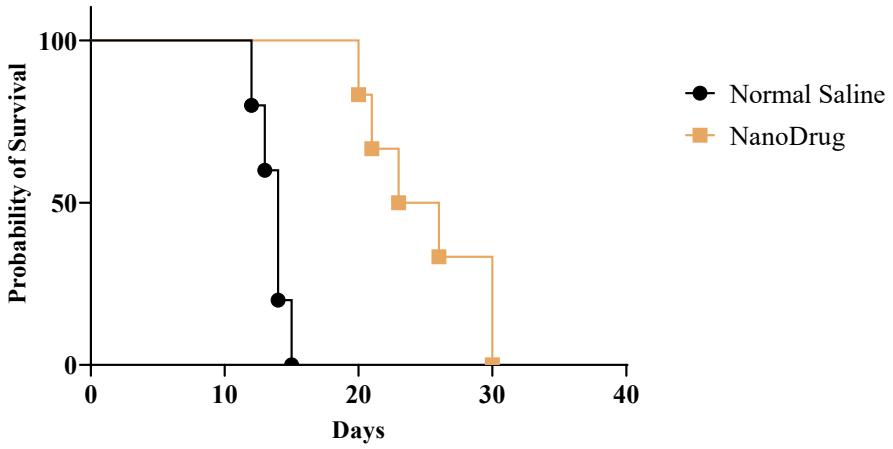

Figure 3. Kaplan-meier diagram to investigate the survival of mice in groups

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Notes: The findings revealed that the mice administered the drug solvent experienced earlier mortality compared to the other group. Two mice from the nano-drug group survived until day 30 without any notable clinical complications. To conduct statistical analysis between the various groups, both the log-rank survival analysis (Mantel-cox) test and the log-rank test for trend were employed.

augmented the infiltration of $CD8^+$ T cells into the tumor microenvironment and consequently enhanced antitumor activity in preclinical studies [28-30]. As a result, anticancer drugs, such as pembrolizumab and ipilimumab, have demonstrated their ability to augment the infiltration of $CD8^+$ T cells into the tumor microenvironment, thereby leading to improved antitumor activity and ultimately better clinical outcomes for cancer patients. In short, the observed increase in $CD8^+$ lymphocytes within the tumor tissue, suggests a beneficial impact of the nanodrug on the immune response against tumors.

In this investigation, as indicated in Figure 3, the modified ZnO nanodrug exhibited a notable impact on the survival rate of the experimental mice, in contrast to the mice that were administered the solvent drug.

Conclusion

The present study provides compelling evidence that the modified ZnO nanodrug effectively suppresses tumor growth and enhances the antitumor immune response. The nanodrug's ability to reduce tumor size and promote the infiltration of $CD8^+$ lymphocytes into the tumor microenvironment underscores its potential as a promising therapeutic strategy for cancer treatment. Moreover, the significant improvement in survival rates observed in mice treated with the nanodrug further corroborates its therapeutic efficacy. These findings warrant further exploration of the modified ZnO nanodrug as a novel and effective anticancer modality.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Shahed University (Code: IR.SHAHED.REC.1401.153).

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Authors' contributions

Study design: Roya Yaraee, Marzieh Eghtedardoost and Sajjad Shekarchian; Preparation of nanomedicine: Zahra Fakhroueian; Consulting: Zahra Fakhroueian; Experimental work: Sajjad Shekarchian and Marzieh Eghtedardoost; Data analysis: Roya Yaraee and Sajjad Shekarchian; Writing: Sajjad Shekarchian; Review & editing: Roya Yaraee, Marzieh Eghtedardoost and Zahra Fakhroueian; Project administration: Roya Yaraee; Funding acquisition: Roya Yaraee and Marzieh Eghtedardoost; Data curation: Roya Yaraee and Marzieh Eghtedardoost; Supervision: Roya Yaraee and Zahra Fakhroueian.

Conflicts of interest

The authors declared no conflict of interest.

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