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Original paper

# The Cytotoxic Effects of Testosterone on iNOS Expression Level in Lung Cancer Cells

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**Abstract**

**Background and Aim:** Association of sex steroids with cancer cells proliferation has been reported in recent studies; however, the findings are still controversial. The present study aimed to determine the cytotoxic effect of testosterone lung cancer (A549) cell and to evaluate the expression level of iNOS gene in A549 cell.

**Method:** A549 cell line was divided into a control group (untreated) and groups treated with 125, 250, 500, and 1000 µg/mL of testosterone. Cell viability was quantified by MTT assay. qRT-PCR was performed to evaluate gene expression level. Flow cytometry was used to assess the apoptosis in cancer cell. Data were analyzed using student’s t-test and ANOVA.

**Results:** The expression level of iNOS gene significantly increased in the A549 cell exposed to a cytotoxic dose of testosterone.

**Conclusion:** In conclusion, the clinical use of testosterone therapy for cancer treatment remains a topic of significant controversy within the medical community. While there is evidence suggesting potential benefits, such as improved quality of life and symptom management, the risks and uncertainties surrounding this approach cannot be ignored. The complex interplay between testosterone and cancer, with the potential for tumor growth and disease progression, necessitates cautious consideration on a case-by-case basis. Further research and clinical trials are essential to better understand the mechanisms involved, identify suitable candidates, and establish safe and effective protocols for testosterone therapy in cancer treatment.

**Keywords:** *Testosterone, A549, Apoptosis*

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## Introduction

Studies have shown that sex hormones play an important role in development of many types of cancer [1], however, they have been reported to have anticancer effects on colon and lung cancer [1], [2]. Lung cancer has been identified as the leading cause of mortality in men and the second leading cause of mortality in women after breast cancer [3], [4].

Although testosterone exerts an inhibitory effect on the growth and development of some types of cancer cells [5], there are evidences suggesting that testosterone can be a contributing factor in the formation of colon tumors [6]. It has also been reported that men tend to have a higher incidence of colorectal cancer than women of similar age [7]. In vitro experimental findings have shown that male steroid hormones can cause adenocarcinomas and stimulate the proliferation of adenocarcinoma cells [8].

Sex steroid hormones, especially testosterone, can stimulate the proliferation of cancer cells or inhibit their growth and proliferation. Sex steroids can also play a role in preventing metastasis or stimulating metastasis in cancer cells [2]. The association between female sex steroids and lung cancer has been reported in women receiving hormone therapy [9]. Blocking the sex steroids receptors by siRNA significantly reduces the proliferation of lung cancer cells *in vitro* [10]. Although previous studies have investigated the effect of testosterone on lung cancer cell, the findings are highly controversial [5], [7], [10], [11]. There are few studies focusing on the cytotoxicity mechanism of testosterone on expression of cluster of inducible nitric oxide synthase (*iNOS*) gene. Considering the effect of testosterone on cell proliferation as well as metastasis of lung cancer cell [5], [12], this study aimed to determine the apoptotic effect of testosterone on A549 cancer cell.

## Material and Methods

This research was carried out at Javid Biotechnology Institute (Tehran, Iran) after obtaining the approval from Institutional Ethics Review Committee of Biomedical Research of Hamedan University of Medical Sciences (Hamedan, Iran) (ID number: IR.UMSHA.REC.1399.342).

*Cell Lines*

A549 cell was obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran).

*Hormone preparation*

Testosterone was obtained from Aburaihan Pharmaceutical Company (Tehran, Iran) and dissolved in dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), and phosphate-buffered saline (PBS) and finally were prepared serially (125, 250, 500, and 1000 μg/mL) [13].

*Cell Cultures*

A549 cell was cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin and kept in a humidified atmosphere with 5% $CO_2$ in a 37°C incubator. The cultured cell with 70-80% confluency was washed with PBS and detached from the flask, using trypsin-EDTA with incubation at 37ºC for 3-4 minutes, followed by addition of the culture medium containing 10% FBS to neutralize the excess trypsin-EDTA activity. The cell suspension was finally centrifuged and the cell pellet was re-suspended in a fresh culture medium for further experiments.

*Cytotoxicity Assay*

The effect of testosterone on cell viability was determined using MTT assay. The cells ($1\times10^4$) were seeded in each well of a 96-well plate for 24 hours. They were treated with different concentrations of testosterone (125, 250, 500, and 1000 μg/mL) for 24 hours and added to wells in eight replicates. The MTT solution (5 mg/mL) (DoBio Biotech, Shanghai, China), diluted in

PBS, was then added to the culture medium (100 λ) and incubated with the cells for three hours at 37˚C in darkness. During this incubation period, water-insoluble formazan crystals were formed and dissolved by adding 100 λ/well of DMSO (Sigma). The optical density (OD) of each culture well was measured at 570 nm, using a microplate reader. The wells containing the culture medium and MTT without the cells were considered as blanks. The $OD_{570}$ of cells without testosterone treatment was considered as 100% viability. The percentage of cell viability (%) was measured as follows [14]:

$$\text{Cell viability (\%)} = (OD_{570\text{-}630} \text{ of treated cells}/OD_{570\text{-}630} \text{ of control cells}) \times 100\%$$

*Real-Time PCR Assay*

A SYBR Green Real-Time Quantitative PCR assay was employed to quantify the expression level of *iNOS* gene after the treatment of A549 cell. Briefly, the cells were seeded into six-well plates ($5\times10^5$ cells/well) and incubated for 24 hours and then exposed to testosterone at final half maximal inhibitory concentrations ($IC_{50}$) (testosterone: 740 μg/mL) and incubated for an additional 24 hours. The total mRNA was extracted from the cells and converted to complementary DNA, using RNA Isolation Kit (RNeasy Plus Mini Kit 50, Qiagen, Valencia, CA, USA) and PrimeScript™ First-Strand cDNA Synthesis Kit (Takara, Tokyo, Japan), respectively, according to the manufacturers' protocols. The primers used for real-time PCR are presented in table 1.

The expression level of target gene was studied using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as internal control. Each amplification reaction was performed in a 20-μL reaction mixture, containing 10 μL of PowerUp 2X SYBR Green PCR Master Mix, 1 μL of each primer (2 μM), 2 μL of cDNA (100 ng) and 6 μL of double-distilled water. After denaturation at 95°C for 15 seconds and 60°C for one minute, amplification was followed by a melting step at 95°C for 20 seconds, at 60°C for 60 seconds and at 95°C for 20 seconds [11]. The expression level of genes was calculated based on the $2^{-\Delta\Delta CT}$ method and normalized to the loading control, GAPDH [13], [15].

**Table 1.** The characteristics of the primers used in the Real-time PCR reaction

| Gene | | Primer sequences |
|---|---|---|
| iNOS | forward: | 5' GTGCCCTGCTTTGTGCG 3' |
| | Reverse: | 5'TCCTCCTGGTAGATGTGGTCCT 3' |
| GAPDH | forward: | 5' CCCACTCCTCCACCTTTGAC 3' |
| | Reverse: | 5' CATACCAGGAAATGAGCTTGACAA 3' |

*Flow Cytometric Analysis of Cell Death*

Differential counting of apoptotic, necrotic, and viable cells was carried out using a fluorescein isothiocyanate (FITC)-Annexin-V/propidium iodide (PI) staining kit (Hoffmann-La Roche Ltd., Basel, Switzerland). This assay involved simultaneous staining with both annexin-V and PI as DNA stains. Three subpopulations of cells were discriminated: (a) PI-negative and FITC-negative viable cells (PI–/FITC–) that maintain the typical asymmetry of plasma membrane lipids; (b) PI-negative and FITC-positive early apoptotic cells (PI–/FITC+) capable of transferring PI outside the cell; and (c) PI-positive and FITC-positive late apoptotic or necrotic cells (PI+/FITC+) with a loss of plasma membrane integrity.

Briefly, the A549 cell ($3\times10^5$ cells/well) was incubated for 24 hours with 200 μg/mL of $IC_{50}$ concentration of testosterone. The trypsinized cells were washed and suspended in an Annexin-V

binding buffer. 5 and 10 µL of FITC Annexin-V and PI solution were added to 100 µL of the cell suspension. Finally, apoptotic, necrotic, and viable cells were analyzed by a flow cytometer [13].

*Statistical Analysis*

Statistical analyses were performed in SPSS version 21.0 (SPSS, Chicago, IL, USA). Differences in the cell viability of the groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Independent samples t-test was used to determine differences in gene expression. All experiments were performed at least three replicates. All data are expressed as Mean±standard deviation (SD) and P-value <0.05 was considered statistically significant.

## Results

*Cytotoxic Effects of Testosterone on the Proliferation of A549 Cell*

The results of MTT assay showed that the viability of A549 cell significantly reduced when treated with 1000 µg/mL of testosterone compared to the control group (P<0.001) (figure 1).

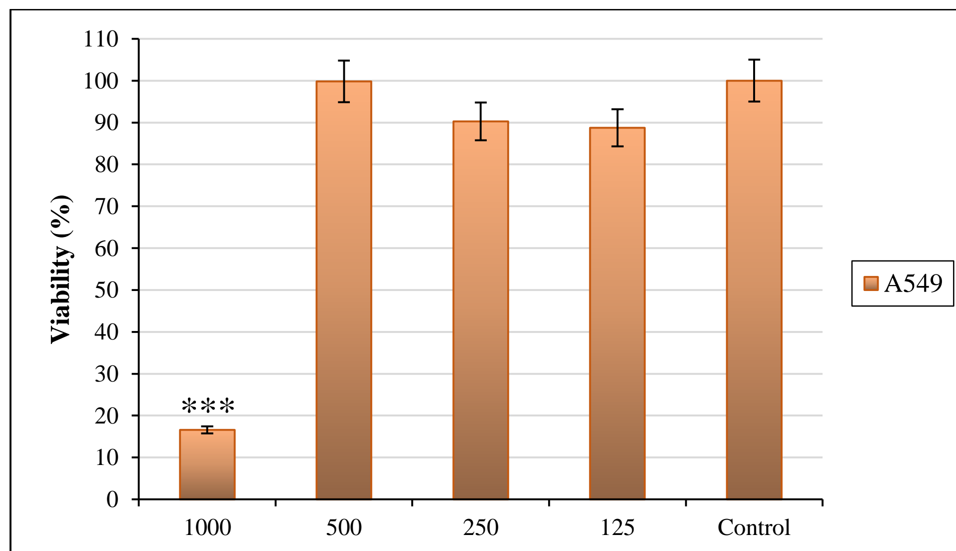


**Figure 1.** Viability of A549 cells after 24h incubation with testosterone. *** represent significant difference compared to control group (***: P<0.001).

*Effects of Testosterone on Expression Levels of iNOS Gene in A549 Cells*

There was a significant increase in iNOS gene expression level (P<0.01) in A549 cell exposed to dose IC50 of testosterone compared with control group (figure 2).

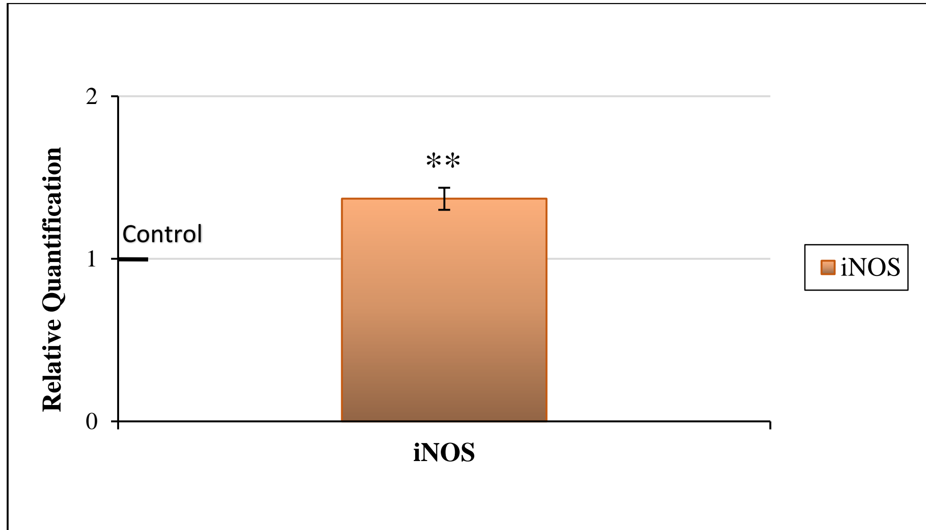


**Figure 2.** Relative expression level (RQ) of iNOs gene in A549 cell exposed to IC50 of testosterone. Ctrl indicates control group=1. * Indicates significant difference compared to control group (**: P<0.01 and ***: P<0.001).

*Evaluation of A549 Cell Apoptosis by Flow Cytometry*

Flow cytometry was used to discriminate early apoptotic cells from late apoptotic and necrotic cells. Figure 3b1 represent the control A549 cells in which there are almost no apoptotic cells.
In testosterone treated A549 cell (figures 3b2) a significant increase in early and late apoptotic cells and a significant decrease in live cells were found and analysis of cell populations indicated distinct sets of population. Annexin V-positive and PI-negative cells increased significantly via treatment of A549 cell with IC50 of testosterone compared to control group, indicating the translocation of PS as an early event in the apoptotic process. Figure 4 shows the live cells, necrosis, early apoptosis, and late apoptosis in control and treated A549 cells.

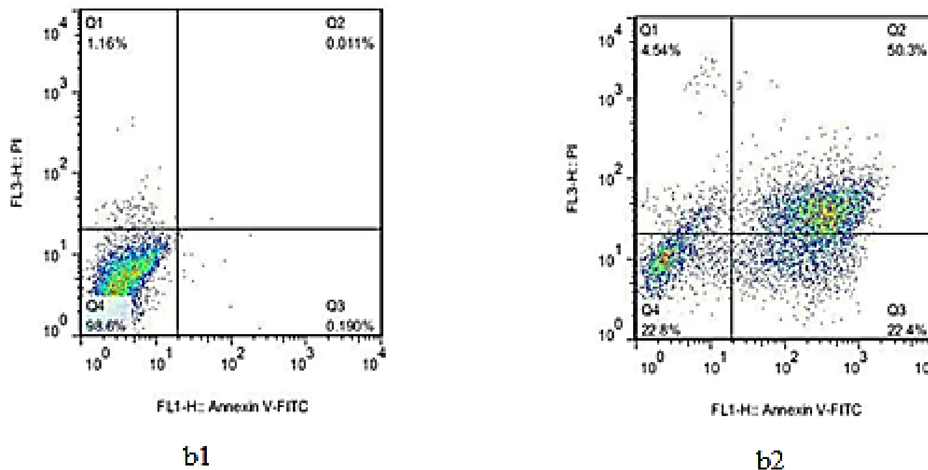


**Figure 3.** Apoptosis in A549 cell line induced by testosterone: Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells. b1: Control non-treated A549 cell; b2: A549 cells treated with IC50 of testosterone.

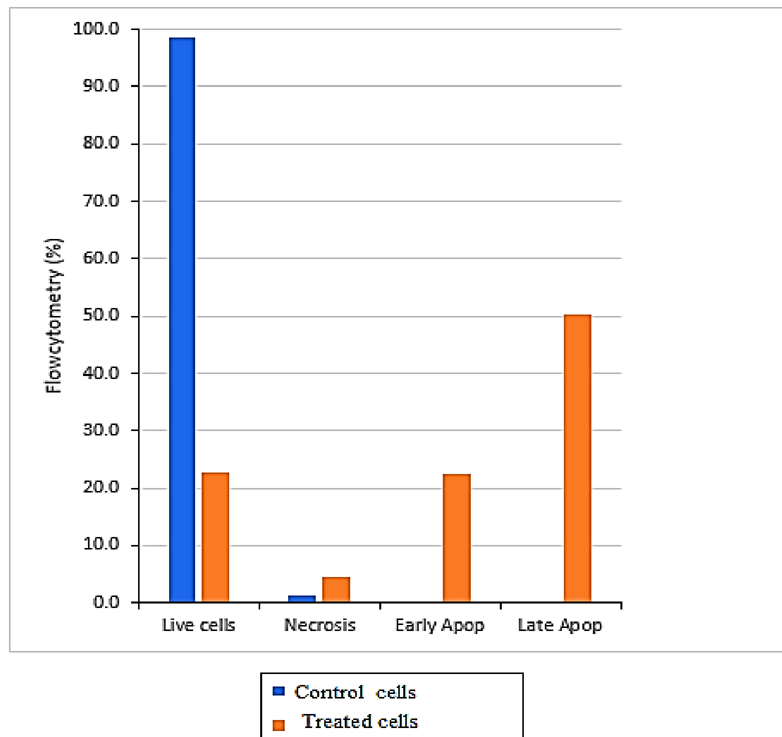


**Figure 4.** Live cells, necrosis, early apoptosis, and late apoptosis in control and treated A549 cells. * and *** represent significant difference compared to control group (*: P<0.05 and ***: P<0.001).

## Discussion

The present results showed that IC50 of testosterone caused a significant reduction in the A549 cell as compared to the control group. A study on the effect of testosterone on the growth and development of some cancer cells showed its inhibitory effect on cancer cells [5].

In line with the present results, some studies have shown that sex steroid hormones can play a role in inhibiting some cancers, such as breast cancer [1]. In contrast to the findings of this study on the cytotoxic effects of testosterone on the survival of A549 cells, studies on testosterone have shown that it can be an effective factor in the development of colon tumors [6]. In line with these findings, another study reported that men are more likely to develop colorectal cancer than women of the same age in the United States [7]. Besides, the results of some studies have shown that male steroid hormones cause adenoma cancers and stimulate the proliferation of adenoma cancer cells [8]. On the other hand, studies on women receiving hormone therapy have reported a higher rate of lung cancer [9].

By examining this gene, it was observed that IC50 of testosterone significantly increased the expression of iNOS gene in the A549 cell. In a study on iNOS, it was found that iNOS is expressed in the brain, breast, lung, and colon tumor cells and that NO can inhibit T-cell proliferation or induce apoptosis, thereby suppressing the immune system [16], [17]. In contrast, another study showed that tumor cell death could be induced by iNOS in cancer cells [18].

## Conclusion

The results of this study showed that the cytotoxic effect of testosterone on the iNOS gene expression significantly increased in the A549 cell. These findings are of great importance, as they

revealed that testosterone hormone, could be used as anti-cancer agents in lung cell. Although this study had some limitations in the research phase, especially in the study of other genes, proteins, and enzymes associated with apoptosis, the present findings can be used in the treatment of lung cancer. However, further cellular, molecular, and clinical research is needed to determine whether testosterone can be effective in treating lung tumors in animal and human models without side effects.

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## Conflict of interests

The authors state that there are no conflicts of interest regarding the publication of this article.

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