ASSOCIATION OF TESTOSTERONE WITH COLORECTAL CANCER (HT29), HUMAN GLIOBLASTOMA (A172) AND HUMAN EMBRYONIC KIDNEY (HEK293) CELLS PROLIFERATION

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Abstract

Testosterone influences cancer development. This *in vitro* experiment was exerted to determine the association of testosterone with human colorectal cancer (HT29), glioblastoma (A172) and human embryonic kidney (HEK293) cells proliferation.

HT-29, A172 and HEK293 cell lines were cultured in standard growth medium, then randomly divided into control group (not exposed to testosterone) and groups exposed to 1, 10, 100 and 1000 µg/mL of testosterone. Cell viability was quantified by MTT assay. Statistical analysis was performed using ANOVA.

Viability of HEK293 cells significantly increased in groups exposed to 1 µg/mL and decreased in groups exposed to 100 and 1000 µg/mL of testosterone compared to control group ($P < 0.05$, $P < 0.05$ and $P < 0.001$, respectively). Viability of HT29 cells significantly increased in groups exposed to 10 and 100 µg/mL of testosterone and significantly decreased when exposed to 1000 µg/mL of testosterone compared to control group ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). Viability of A172 cells significantly decreased in groups exposed to 100 and 1000 µg/mL of testosterone compared to control group ($P < 0.001$).

In conclusion, different doses of testosterone have enhancing or suppressive effects on HEK293, HT29 and A172 cells proliferation; according to which, considering clinical use of testosterone therapy for cancer treatment is a highly controversial issue.

Key words: Testosterone, HT-29, A172, HEK 293, Proliferation.

INTRODUCTION

Testosterone is the main androgen required for development of male sex organs and it plays an important role in maintaining sex drive, sperm production, muscle strength and bone mass. In males, the majority of testosterone is secreted from the testes

and in females half of the testosterone is produced by the ovaries and adrenal glands (1). HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology and it has retained certain characteristics of the normal tissue, such as hormone receptors (2). HEK293 cells were generated by adenovirus transformation of human embryonic kidney cells since 25 years ago (3) and have been one of the most widely used immortalized normal cell lines in biomedical research. A172 is human glioblastoma cell line derived from glioblastoma removed from a 53 year old male.

Among cancers, colorectal cancer is one of the leading causes of cancer-related morbidity and mortality. The vast majority of colorectal cancers are classified as adenocarcinomas, which begin in the mucus-making glands lining the colon and rectum (4). Colorectal cancer is the second most commonly diagnosed cancer in women and the third in men worldwide. The rates vary more than 10 times across the world; high-income countries have approximately 2.5 times higher rates than do low-income countries (5).

Glioblastoma, or astrocytoma WHO grade IV is the most common and most aggressive fatal primary brain cancer found in humans. Most glioblastomas manifest rapidly *de novo*, without recognizable precursor lesions. They typically occur in middle age to elderly patients, with brief clinical history, rapid progression and short overall survival time (6).

Experimental and epidemiological data support a major role for sex steroid hormones in the pathogenesis of several cancers (7), manifesting a series of important links between human endocrine system and cancer development. The role of endogenous sex hormones in progression or prevention of digestive system cancers has also been studied in recent years. Several sex hormone receptors, including androgen, progesterone, and estrogen receptors, are expressed

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in the digestive tract cells (8) which can support the possible effects of sex steroid hormones on cancer development in the digestive system. In this regard, although colorectal cancer is generally not considered to be a hormone-related malignancy, accumulating evidence has suggested that sex hormones are relevant to its development (9, 10).

Testosterone may also play a role in the development of brain tumors and/or controlling their growth. Evidences have demonstrated that androgen receptors have a broad distribution in the brain (11), supporting a role for androgens in brain disorders. It has also been reported that sex steroids may have anti-tumor effects on glioblastoma cells (12). Although sex hormones influence the development and growth of brain tumors (13), a study has shown that sex steroid hormone usage was not associated with glioma risk (14).

There are evidences suggesting that testosterone may actually be a contributing factor in the formation of colon cancer tumors (15). In line with these findings, it has been shown that men tend to have a higher incidence rate of colorectal cancer than women of similar age in the US (16); this may come from the role played by androgens in the development of colorectal cancer. However, a small prospective study has reported that higher circulating levels of dehydroepiandrosterone sulfate (DHEAS), an androgen precursor, were associated with a lower risk for colon cancer (17).

Despite research indicating that testosterone may cause different types of cancer, there are studies showing that testosterone has protective effects against cancer development in various tissues (9, 18-23). The relationship between testosterone and development of cancer still remains as a controversial issue. Moreover, in recent years a large amount of research has focused on the link between testosterone levels and cancer risk or prevention, but there is limited evidence about the effects of exogenous testosterone on proliferation of cancer cells at cellular and molecular level. The focus of this *in vitro* research was to investigate the association of different doses of testosterone with colorectal cancer, glioblastoma and human embryonic kidney cells proliferation. Given the considerable attention in the dose-response relationships for testosterone as a carcinogen, evaluating its cytotoxicity on colorectal cancer and glioblastoma cells compared to non-cancerous HEK293 cells, is a critical first step in designing experiments for understanding testosterone role in development of colorectal cancer and glioblastoma.

MATERIALS AND METHOD

Cell line

HT-29, A172 and HEK293 cell lines were obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran).

Testosterone

Testosterone Enanthate was obtained from Abu Reyhan Pharmaceutical Company (Tehran-Iran) and solved in DMSO (0.1%), toyeen and PBS to produce different concentrations (1, 10, 100 and 1000 µg/mL) of testosterone.

Cell culture

The HT-29, A172 and HEK293 cells were maintained in complete growth medium (CGM) supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin). The cells (1×10^6 cells/mL) were plated in T-25 flasks containing 5 mL of CGM and grown in a humidified incubator under an atmosphere of 95% air and 5% CO₂ at 37°C to subconfluence (90 - 95%). The culture medium was replaced every 48 hours. Once the cells reached 90 - 95% confluency, the medium was aspirated and the cells monolayer was washed three times with sterile phosphate buffered saline. The cell monolayer was treated with 1 mL of 0.25% (w/v) trypsin-EDTA and incubated briefly at 37°C and visualized microscopically to ensure complete cell detachment. Cells were re-suspended in complete growth medium and stained with trypan blue (100 µL of cell suspension and 100 µL of 0.4% trypan blue), incubated for 2 minutes at room temperature, and counted using a hemacytometer. The cells were seeded at a density of 1×10^4 cells/well in 96-well microtiter tissue culture plates prior to testosterone treatment.

Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed for assessing viability of HT-29, A172 and HEK293 cells exposed to 1, 10, 100 and 1000 µg/mL of testosterone 24 hours after incubation. MTT assay was performed according to standard protocols (24). Cells were seeded in 96-well plates with 1×10^4 cells/well and placed at 37°C in a 5% CO₂ humidified incubator until 60% confluency.

The complete growth medium was removed and the cells were serum-starved for 24 h prior to treatment. Cells incubated in culture medium alone (untreated cells) served as control group. In experimental groups, the cells were treated with 1, 10, 100 and 1000 µg/mL

of testosterone for 24 h. The medium was then removed and 100 μ L of MTT solution (5 mg/mL in sterile H₂O) was added to each well. The plates were incubated under 95% atmosphere air and 5% CO₂ at 37°C. The MTT solution was removed and 200 μ L aliquots of DMSO were added to each well to dissolve the formazan crystals followed by incubation for 10 min at 37°C. Treatments were performed in triplicates, and optical densities were read at 540 nm by spectrophotometric method.

Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA method) followed by post hoc Tukey's multiple comparisons test in SPSS 20 software. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Figure 1 shows the viability of HEK293 cells exposed to 1, 10, 100 and 1000 μ g/mL of testosterone enanthate. Viability of HEK293 cells significantly increased in groups exposed to 1 μ g/mL of testosterone compared to control cells ($P < 0.05$). However, there was no significant difference between viability of HEK293 cells exposed to 10 μ g/mL of testosterone compared to control cells. The cell viability significantly decreased in HEK293 cells exposed to 100 and 1000 μ g/mL of

Figure 1. Viability of Normal Embryonic Kidney (HEK293) Cells Exposed to 1, 10, 100 and 1000 μ g/mL of Testosterone in Cell Culture. OD (optical densities) of viable cells were measured at a wavelength of 540 nm representing cell viability. *, #, †, and Θ indicate significant difference compared with control, 1, 10, 100 and 1000 μ g/mL of testosterone receiving groups, respectively. NS indicates non-significant difference. (*: $P < 0.05$, **: $P < 0.001$, ###: $P < 0.001$, ††: $P < 0.01$, †††: $P < 0.001$, ΘΘΘ: $P < 0.001$).

testosterone compared to control cells ($P < 0.05$ and $P < 0.001$, respectively). There was also significant difference in cell viability between HEK293 cells exposed to 100 and to 1000 μ g/mL of testosterone ($P < 0.001$).

Figure 2 shows the viability of HT-29 cells exposed to 1, 10, 100 and 1000 μ g/mL of testosterone enanthate. There was no significant difference between viability of HT-29 cells exposed to 1 μ g/mL of testosterone and control cells. However, the cell viability significantly increased in HT-29 cells exposed to 10 and 100 μ g/mL of testosterone compared to control cells ($P < 0.05$ and $P < 0.001$, respectively). There was also significant difference in cell viability between HT-29 cells exposed to 10 and 100 μ g/mL of testosterone ($P < 0.05$). Exposure of HT-29 cells to 1000 μ g/mL of testosterone led to a significant decrease in viability of HT-29 cells ($P < 0.001$).

Figure 3 shows the viability of A172 cells exposed to 1, 10, 100 and 1000 μ g/mL of testosterone enanthate. There was no significant difference between viability of A172 cells exposed to 1 and 10 μ g/mL of testosterone and control cells. However, the cell viability significantly decreased in A172 cells exposed to 100 and 1000 μ g/mL of testosterone compared to control cells ($P < 0.001$). There was also significant difference in cell viability between A172 cells exposed to 100 and 1000 μ g/mL of testosterone ($P < 0.001$).

Figure 2. Viability of Colorectal Cancer (HT-29) Cells Exposed to 1, 10, 100 and 1000 μ g/mL of Testosterone in Cell Culture. OD (optical densities) of viable cells were measured at a wavelength of 540 nm representing cell viability. *, #, †, and Θ indicate significant difference compared with control, 1, 10, 100 and 1000 μ g/mL of testosterone receiving groups, respectively. NS indicates non-significant difference. (*: $P < 0.05$, **: $P < 0.001$, ###: $P < 0.001$, ††: $P < 0.01$, †††: $P < 0.001$, ΘΘΘ: $P < 0.001$).

Figure 3. Viability of Glioblastoma (A172) Cells Exposed to 1, 10, 100 and 1000 µg/mL of Testosterone in Cell Culture. OD (optical densities) of viable cells were measured at a wavelength of 540 nm representing cell viability. *, #, †, and Θ indicate significant difference compared with control, 1, 10, 100 and 1000 µg/mL of testosterone receiving groups, respectively. NS indicates non-significant difference. (***:P<0.001, ####:P<0.001, ††:P<0.01, †††:P<0.001, ΘΘ:P<0.01, ΘΘΘ:P<0.001).

DISCUSSION

Our findings indicated that cell viability increases in human embryonic kidney cells exposed to low testosterone concentration (1 µg/mL); however, exposure of human embryonic kidney cells to higher concentrations of testosterone (100 and 1000 µg/mL) gives rise to decreased cell viability, demonstrating that the effect of testosterone on human embryonic kidney cells is dose-dependent. In line with our finding, there are studies showing that testosterone may promote or inhibit proliferation of non-cancerous cells. It has been shown that androgen receptor is expressed in kidney cells and the androgen receptor signalling has been found to have important roles in modulating kidney cells proliferation (25). In addition, testosterone increases the proliferation of mesenchymal stem cells (26) and promotes skeletal muscle myoblast cell line proliferation in the rat (27). By contrast, there are reports indicating that androgens can inhibit proliferation of endothelial (28), human renal tubular (18) and vascular smooth muscle non-cancerous cells (21). It has also been shown that primary cell lines of dermal papilla cells from human show different responses to androgens based on androgen concentrations (29).

We have also shown in our study that HT-29 cells viability increases in response to 10 and 100 µg/mL of testosterone; however, exposure of HT-29 cells to higher concentration (1000 µg/mL) of testosterone led to a significant decrease in viability of HT-29 cells. Our finding reveals that different doses of testosterone may have enhancing or suppressive effects on colon cancer cells proliferation. Previous studies have also reported conflicting data on the effects of testosterone on colon cancer development (30). There are observational and clinical evidences indicating that sex hormones influence development of colorectal cancer in men and women; however, the relationship between endogenous sex hormone levels and colorectal cancer is unclear (31). It has been shown orchidectomy in rats leads to a drastic decrease in colon cancer susceptibility (10), indicating that low levels of testosterone have a potential role in development of colon cancer. In addition, results of a prospective study have revealed that testosterone has an important role in colorectal cancer development in men (9). Testosterone also stimulates growth of colon cancer cells *in vitro*, a mechanism that can be halted with anti-androgens (32). There are also findings showing that a higher circulating level of androgen precursor is associated with a lower risk for colon cancer (17), supporting our finding. However, in contrast to our findings, there are reports showing that high levels of testosterone may enhance the risk of developing cancer (33). Despite attempts to clarify the effects of testosterone on cancer development, the relationship between endogenous testosterone and development of cancer still remains highly controversial (34).

Our findings have also shown that the viability of A172 cells significantly decreases in response to higher concentrations (100 and 1000 µg/mL) of testosterone; however, it does not significantly change in response to lower concentrations (1 and 10 µg/mL) of testosterone. In line with our finding, it has been shown that sex steroids play a role in the development of glioblastoma. The existence of testosterone receptors in different parts of the brain (11) indicates that testosterone can influence brain cells development. Some glioblastomas also express aromatase, the enzyme responsible for the conversion of testosterone to estradiol, revealing that glioblastoma cells have a potential to respond to testosterone (35). Previous studies have demonstrated that androgens directly exert neuroprotective effects on brain cells (36). Research has also shown that sex steroids may have anti-tumor effects on glioblastoma cells (12). However, and in line with our finding,

there are experimental data indicating that there is no relationship between weak androgens and glioblastoma development (37). In contrast to our finding there is a report suggesting that androgens may promote growth of glioblastoma (38).

In conclusion, different doses of testosterone have enhancing or suppressive effects on non-cancerous human embryonic kidney, colon cancer and glioblastoma cells proliferation *in vitro*; according to which, considering clinical use of testosterone therapy for cancer treatment is a highly controversial issue. Further research is also needed to clarify the effects of testosterone on colon cancer and glioblastoma cells at cellular and molecular level.

Conflict of interest

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

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