

Effect of letrozole combined with testosterone on the proliferation and apoptosis of endometrial carcinoma cells

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Summary

Objective: To investigate the effect of the androgen testosterone on Ishikawa endometrial cancer (EC) cells in the presence of the aromatase inhibitor letrozole. **Methods:** Cultured Ishikawa EC cells were divided into three treatment groups: testosterone plus letrozole, testosterone alone and letrozole alone. The respective groups were treated for 48 h with these agents at final concentrations of 0.5, 1, 1.5, 2 or 2.5 $\mu\text{mol/L}$. The effect of combined letrozole and testosterone treatment was determined by MTT assay and flow cytometry. The effects of combined treatment on cell proliferation and apoptosis were also measured, while the concentration of estrogen in the culture solution was measured after treatment at a concentration of 2.5 $\mu\text{mol/L}$. **Results:** Inhibition of proliferation in the testosterone plus letrozole group increased with the concentration of these agents, with maximal inhibition observed at 2.5 $\mu\text{mol/L}$. Similarly, the rate of apoptosis increased with increasing concentration in the combined testosterone plus letrozole group. The proliferation and apoptosis rates were different between the three different testosterone and letrozole groups, while the estrogen concentration of the testosterone plus letrozole group was significantly lower than that of the testosterone group. **Conclusion:** The androgen testosterone has anti-proliferative and pro-apoptotic effects on EC cells.

Key words: Endometrial cancer; Androgen; Antitumor; Proliferation; Apoptosis.

Introduction

Endometrial cancer (EC) is one of the most common female genital tract tumors, with the risk increasing after the age of 60 years. The incidence rate for EC has gradually increased and it has also been observed in younger patients [1]. However, there has been little progress in improving the treatment of EC. Faced with the increasing incidence and mortality from this cancer type [2], it is imperative to find more effective treatment methods and therapeutic indicators.

Previous research suggests that steroid hormones play a central role in the etiology of EC. The risk of postmenopausal EC is related to serum concentrations of total testosterone, free testosterone, estrone, estradiol and free estradiol [3]. There is evidence that EC is mainly associated with high levels of estrogen, prolonged stimulation and the lack of progesterone. EC has also been linked to resistance to estrogen [4]. There is some evidence to suggest the estrogen level could be a marker for the clinical features and risk of recurrence of EC [5]. However, the relationship between EC and androgen has not been studied in detail.

In postmenopausal patients, estrogen is mainly derived from androgen, which is secreted by adrenal and ovarian glands that are stimulated by aromatase [6]. Androgen is a direct precursor of estrogen and thus it is difficult to distinguish the androgenic effects on EC [7]. After adjusting for the European Prospective Investigation into Cancer and Nutrition (EPIC) residual factor calculation, researchers have

found that steroid actions were mainly driven by estrogen, and that androgen itself did not increase the risk for EC. Androgen does not stimulate normal endometrial cell proliferation and is negatively correlated with the risk of EC [8]. In the present *in vitro* study we investigated the effect of the androgen testosterone on Ishikawa EC cells by blocking its conversion to estrogen using the aromatase inhibitor letrozole.

Materials and Methods

Experimental Materials

The Ishikawa EC cell line, which responds to steroid hormones, was purchased from Nanjing Kaiji Biotechnology Development Co., Ltd. Letrozole was purchased from MadChemExpress, USA. Testosterone was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. The MTT cell proliferation kit and Annexin V-FITC/PI apoptosis kit were purchased from Nanjing Kaiji Biotechnology Development Co., Ltd.

Cell Culture and Treatment

Ishikawa EC cells were routinely cultured in RPMI-1640 medium containing 10% high quality calf serum. Cells in the logarithmic growth phase were used for subsequent experiments. Testosterone and letrozole were dissolved in DMSO to make a stock solution of 50 $\mu\text{mol/L}$. This was diluted in RPMI-1640 complete medium to the required final concentrations of 0.5, 1, 1.5, 2 and 2.5 $\mu\text{mol/L}$. Letrozole and testosterone were prepared in a ratio of 1:1 for the testosterone plus letrozole group.

Effect on Cell Proliferation

The MTT method was used to evaluate the effect on cell proliferation. After washing cells in the logarithmic growth phase with PBS, trypsin (0.25%) was added for digestion and the cells were collected, counted and the final cell concentration was adjusted. Each well had a volume of approximately 100 μ L and the 96-well plate was seeded at a density of 1×10^5 cells/ml. After plating, the cells were cultured in a 37 °C incubator for 24 hours. The cells were divided into three treatment groups: testosterone, letrozole and testosterone plus letrozole. The culture solution was discarded and testosterone, letrozole, or testosterone plus letrozole were added in 100 μ L volumes to give final drug concentrations of 0.5, 1, 1.5, 2 or 2.5 μ mol/L. The cell culture was repeated to set up a blank group and a control group. The control group contained an equal volume of complete medium and the same amount of cells as above, whereas the blank group contained only complete medium without cells. Every 24 h, 6 wells were selected from the testosterone plus letrozole group and 20 μ L of MTT solution with a volume fraction of 0.5% was added to each well. Following incubation for 4 hours, 150 μ L of DMSO solution was added and the cells were shaken at low speed for 10 min. After the purple crystals were completely dissolved, the OD value of the cells was measured at a wavelength of 550 nm using a fully automatic microplate reader. The cell proliferation rate was calculated as follows: cell proliferation rate = OD550 of the experimental group cells / OD550 of the control cells \times 100%. The cell proliferation inhibition rate was measured after 24 h, 48 h and 72 h growth in the testosterone plus letrozole group. The 48 h time gave the most consistent results and hence this incubation period was selected to study the testosterone and letrozole alone groups.

Effect on Apoptosis

Cells growing in the log phase were washed with PBS, digested with 0.25% trypsin and then collected by centrifugation. A single cell suspension was made to a density of 3×10^5 cells / ml and seeded into a 6-well plate at approximately 2 mL per well. After being cultured for 24 hours in a 37 °C incubator, cells were divided into the three treatment groups. Testosterone, letrozole, or testosterone plus letrozole were added at final concentrations of 0.5, 1, 1.5, 2 or 2.5 μ mol/L. The same volume of complete medium was added to the control group. After incubating for 48 h, the cells were digested with 0.25% EDTA-free trypsin and washed 3 times with precooled PBS. After centrifugation, the cells were added to a flow tube and 500 μ L of binding buffer solution, 5 μ L of FITC-labeled Annexin V mixture and 5 μ L of Propidium Iodide solution were added. After mixing, the cells were incubated for 30 min in the dark. Binding buffer (400 μ L) was then added and apoptosis was detected by performing flow cytometry within 1 h. This experiment was repeated three times in order to calculate the apoptotic rate for each treatment group.

Table 1. — Inhibition of cell proliferation in the testosterone plus letrozole group at different times and concentrations (% , mean \pm SD)

Group	24 h	48 h	72 h
0.5 μ mol/L	2.30 \pm 1.11 ^a	7.70 \pm 0.49 ^{ab}	1.02 \pm 0.63 ^{abc}
1.0 μ mol/L	10.32 \pm 2.27 ^a	8.91 \pm 0.62 ^{ab}	1.95 \pm 0.71 ^{abc}
1.5 μ mol/L	15.71 \pm 1.39 ^a	11.29 \pm 1.21 ^{ab}	3.59 \pm 1.91 ^{abc}
2.0 μ mol/L	19.36 \pm 4.16 ^a	13.63 \pm 1.44 ^{ab}	3.65 \pm 2.27 ^{abc}
2.5 μ mol/L	40.80 \pm 1.59 ^a	17.49 \pm 0.45 ^{ab}	4.15 \pm 2.19 ^{abc}
Control group	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00

a, compared with the control group for the same time; b, compared with the same concentration for 24 h; c, compared with the same concentration for 48 h

Estrogen Concentration

The estrogen concentration in the culture medium of each of the three treatment groups (2.5 μ mol/L treatment for 48 h) was compared after detection using electrochemiluminescence.

Statistical Analysis

SPSS19.0 statistical software was used. The cell proliferation and apoptosis rates were analyzed by Mauchly's "spherical symmetry" test and repeated measures analysis of variance. The groups were compared to satisfy the normal distribution selection t-test. Variance analysis was performed for comparisons between groups with a normal distribution. $p < 0.05$ was considered statistically significant and data was expressed as the mean \pm standard deviation (mean \pm SD).

Results

Effect on Cell Proliferation

The MTT assay was used to evaluate the effects on cell proliferation in the testosterone plus letrozole treatment group after 24 h, 48 h and 72 h incubation (Table 1). Using repeated measures data analysis of variance and Mauchly's sphericity hypothesis test, the data conformed to sphericity with $W = 0.850$ and $p = 0.064$. A high correlation was observed between the inhibition of cell proliferation and time of culture, and with the concentration of testosterone plus letrozole. Inhibition of proliferation increased with time ($F = 820.00$, $p < 0.01$). In addition, inhibition of proliferation increased with increasing concentration of testosterone plus letrozole ($F = 254.30$, $p < 0.01$). Overall, inhibition increased with time from 24 h to 72 h treatment at each concentration ($F = 146.55$, $p < 0.01$), with the difference being statistically significant. The difference within the 24 h group was large, but within the 72 h group it was not so obvious. Compared to the controls and to the 24 h group, the 48 h group showed a progressive increase in inhibition with increasing concentration. This time of incubation was therefore selected as the treatment time for follow-up experiments.

Table 2. — Inhibition of Ishikawa cell proliferation after 48 h of treatment (% mean \pm SD)

Group	LT	L	T
1.0 nmol/L	7.73 \pm 3.01	5.09 \pm 0.48 ^a	13.18 \pm 0.46 ^b
1.5 nmol/L	8.98 \pm 0.62	4.90 \pm 0.61 ^a	10.28 \pm 0.72 ^b
2.0 nmol/L	11.34 \pm 1.19	5.18 \pm 0.32 ^a	7.78 \pm 0.73 ^b
2.5 nmol/L	13.67 \pm 1.45	4.89 \pm 0.29 ^a	4.34 \pm 0.41 ^b
3.0 nmol/L	17.51 \pm 0.43	5.26 \pm 0.74 ^a	2.95 \pm 0.32 ^b
Control group	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00

a, compared with the testosterone plus letrozole group, $p < 0.05$;

b, compared with the testosterone plus letrozole group, $p < 0.05$

Inhibition of Ishikawa cell proliferation was investigated in each experimental treatment group after 48 h of incubation (Table 2). No significant inhibition in cell proliferation was observed for the letrozole group (Group L) ($F = 1.975$, $p = 0.120$, $p > 0.05$). In the testosterone group (Group T), cell inhibition decreased significantly with increasing concentration ($F = 6.507$, $p = 0.001$). As observed earlier, the inhibition of cell proliferation in the testosterone plus letrozole group increased significantly with increasing concentration ($F = 845.17$, $p < 0.05$). Inhibition of cell proliferation was significantly different between the treatment groups, with testosterone plus letrozole being significantly higher than testosterone alone or letrozole alone ($F = 845.17$, $p < 0.05$).

Effect on Apoptosis

The three different treatment groups were incubated at 2.5 $\mu\text{mol/L}$ for 48 h to evaluate the effects on apoptosis. The observed apoptotic rates (mean \pm SD) for testosterone plus letrozole, letrozole alone, testosterone alone and control groups were 16.1 \pm 1.07, 10.16 \pm 2.0, 11.5 \pm 0.78, and 7.1 \pm 1.3, respectively. Compared to the control group, apoptosis rates in the letrozole and testosterone alone groups showed a non-significant increase ($p > 0.05$) (Figure 1). The apoptosis rate observed for the testosterone plus letrozole group was significantly higher than either the letrozole or testosterone alone groups ($p < 0.05$). Apoptotic rates (mean \pm SD) for the testosterone plus letrozole group at concentrations of 0.5, 1, 1.5, 2 and 2.5 $\mu\text{mol/L}$ were 11.16 \pm 1.15, 15.03 \pm 0.8, 15.20 \pm 1.00, 16.16 \pm 1.07 and 19.90 \pm 1.47, respectively. The apoptotic rate increased significantly with increasing concentration ($F = 43.70$, $p < 0.01$).

Estrogen Concentration

Cell cultures were grown for 48 hours in the presence of 2.5 $\mu\text{mol/L}$ of each treatment and the culture medium then evaluated for estrogen concentration. This was measured as 84.6 \pm 2.96 pg/ml in the testosterone group, 67 \pm 0.7 pg/ml in the testosterone plus letrozole group, 53.2 \pm 4.6 pg/ml in the letrozole group, and 70.8 \pm 8.43 pg/ml in the control group. The estrogen content in the testosterone group was significantly higher than in each of the other three groups ($F = 32.629$, $p < 0.05$).

Discussion

It is currently believed that androgens have two types of action on EC. In the first, aromatase converts androgen to estrogen, which is then thought to promote the development and progression of EC. Under the action of aromatase, the estrogen-related symptoms caused by high concentrations of androgens have an impact on EC. For example, the high androgen symptom of polycystic ovary syndrome (PCOS) is consistent with abnormal proliferation of endometrial cells, and thus PCOS is a high risk factor for EC [9]. The other type of action is that of androgen acting directly on EC cells to affect their proliferation and apoptosis. In previous cases of transgender patients transitioning from female to male, it was found that testosterone plays an anti-proliferative role in the endometrium, thereby reducing the risk of EC [10]. There are currently very few studies on the role of androgens in gynecological tumors. We cannot infer from this study that promotion of EC growth is caused by estradiol produced by aromatization of androgens, or from the direct action of testosterone without altering the level of estradiol.

In a case-control study, Clendenen *et al.* measured the relationship between testosterone, dihydrotestosterone, DHEAS, DHEA and other androgens in the serum and plasma of premenopausal women, with the aim of determining the relationship between androgen concentrations and EC risk. These researchers found a trend for increased risk of EC with increasing testosterone concentration, but this did not reach statistical significance [11]. When testosterone is added to EC tissue, the aromatase mRNA content increases [12]. Moreover, Feng Wen *et al.* reported that androgen expression in EC was negatively correlated with the International Federation of Gynecology and Obstetrics (FIGO) stage and the depth of myometrial invasion. The role of testosterone in EC may therefore be related to its conversion into estrogen by aromatase, with testosterone itself not having a direct promoting effect. Estrogen concentration is a predictor of the clinical features and risk of recurrence of EC, and elevated endogenous estrogen may support the proliferation of disseminated cancer cells [13]. It is difficult to distinguish whether androgen, a substrate for the synthesis of estrogen precursors, has an independent effect on EC cells [14]. Studies by EPIC have actually shown a negative correlation between androgen and EC risk [15]. Therefore, androgens such as testosterone may play a more complex role in the development and progression of EC.

The synthetic precursor of estradiol and its metabolites are thought to be associated with EC characteristics. To explore the relationship between testosterone and risk of EC, this study examined the effect of testosterone plus letrozole on the proliferation of EC cells. The growth inhibition of Ishikawa EC cells increased significantly with increasing concentration of these agents, whereas the inhibition decreased with increasing concentration of testosterone alone. The inhibition of cell proliferation observed in the

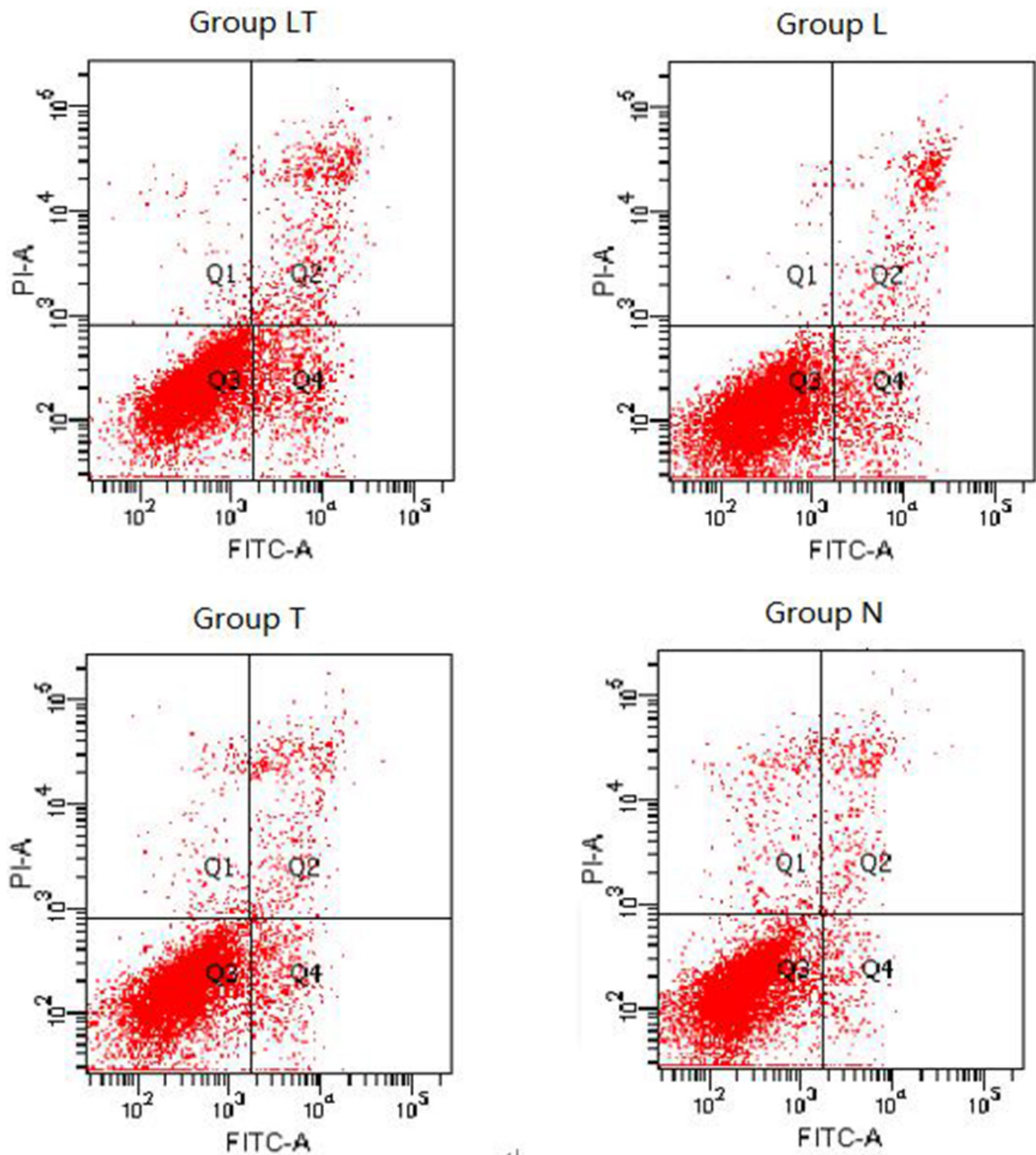


Figure 1. — Apoptotic rates in Ishikawa cells for different treatment groups

testosterone plus letrozole group was significantly different to that of the testosterone and letrozole alone groups. This suggests the effect of estrogen was excluded and that testosterone-independent actions inhibited the proliferation of Ishikawa EC cells. As the concentration of testosterone plus letrozole increased, the rate of apoptosis increased significantly when compared with the letrozole, testosterone and control groups. Together, these results indicate that the

combination of testosterone plus letrozole within a certain concentration range has anti-proliferative and pro-apoptotic effects on EC cells compared to letrozole alone and testosterone alone. In the testosterone plus letrozole group, under the inhibition of letrozole conversion, testosterone was independently applied to the Ishikawa EC cells and both the inhibition of proliferation and apoptosis rates increased with increasing testosterone concentration. In contrast to

the increased estradiol concentration observed in the testosterone group, no significant increase was seen in the testosterone plus letrozole group. This suggests the proliferative effect of testosterone was achieved by estradiol, with androgen itself inhibiting proliferation and promoting apoptosis. We conclude that after treatment with letrozole, testosterone may inhibit proliferation and promote apoptosis of EC cells, and the control of estrogen levels may also reduce the risk of EC onset.

In recent years, the role of the orphan nuclear receptor DAX-1 in EC has received increasing attention. Studies have shown that the androgen receptor (AR) can downregulate transcription of the cell cycle-dependent kinase cycling D1 by DAX-1 after activation by androgen. Afterwards, tumor cells are blocked at the G1 phase, proliferation is slowed and apoptosis is increased. There is also evidence that DAX-1 has a negative regulatory effect on steroid hormones in EC cells. The immunoreactivity of DAX-1 was inversely correlated with the histological grade of EC and was significantly higher in normal, highly proliferating endometrial tissues compared to EC tissues. This evidence therefore supports a role for testosterone in the anti-proliferation and pro-apoptosis of EC cells.

Conclusion

Previous studies have focused on blocking the conversion of androgens to estrogens, while little attention has been paid to the effects of androgens on EC. This study used the aromatase inhibitor letrozole and the androgen testosterone in an EC cell culture model. Under the action of letrozole, testosterone conversion to estradiol was blocked and hence an androgen-independent effect was established. In this EC cell model, androgens have the effect of inhibiting cell proliferation and promoting apoptosis. Therefore, androgens may play a role in the treatment of EC and in predicting its efficacy. The specific mechanism of action of androgen and the full range of its effects requires further exploration.

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Conflict of interest

All authors declare no conflict of interest and gave their consent for publication.

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