

Qianlongtong Inhibits Proliferation and Induces Apoptosis of Hyperplastic Prostate Cells

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Abstract

Qianlongtong is a compound made from traditional Chinese herbs and it has proven to be very effective to treat patients with benign prostate hypertrophy. However, its mechanism is still unknown. This study is designed to investigate the effect of Qianlongtong on proliferation and apoptosis of hyperplastic prostate cells. Flow cytometry (FCM) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were used to assess proliferation and apoptosis of hyperplastic prostate cells in the following groups: control group, tamoxifen group, and groups with low, moderate, and high dosage of Qianlongtong. Reverse transcription-polymerase chain reaction analysis was used to investigate the underlying mechanisms for increased apoptosis. Cells treated with Qianlongtong were mainly blocked in the G0/G1 phase. The apoptotic index of each group was significantly higher than that in the control group. The apoptotic index in the high- and moderate-dosage groups was similar to that in the tamoxifen group. The high- and moderate-dosage groups had lower Bcl-2 and higher Bax messenger RNA (mRNA) levels compared with the control group. Qianlongtong inhibits proliferation and promotes the apoptosis of hyperplastic prostate cells.

Keywords

Qianlongtong, BPH, apoptosis

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Benign prostatic hyperplasia (BPH) is the most common benign disease in middle-aged and elderly men with dysuria (Bushman, 2009). Its pathological features include hyperplasia of prostate epithelial and stromal cells and enlargement of prostate gland volume. The pathogenesis of BPH is very complex. It is commonly accepted that androgen is one of the critical players in this process, since it orchestrates the proliferation and differentiation of prostate epithelial and stromal cells. In recent years, it has been found that growth factors such as insulin-like growth factor (IGF), fibroblast growth factor (FGF), as well as transforming growth factor (TGF) might be other crucial players in the pathogenesis of BPH (La Vignera, Condorelli, Russo, Morgia, & Calogero, 2016). The main clinical manifestations of BPH are urinary frequency and urgency, hesitancy, straining, low urinary stream, weak urine flow, terminal dripping, prolonged voiding time, urinary retention, and urgent or overflow incontinence, and so forth. In addition, BPH can also cause secondary

hematuria, urinary tract infection, bladder calculi, hydrups of upper urinary tract, renal dysfunction, and other serious complications. At present, the main lines of treatment for BPH are observation, medication and surgery. Alpha-receptor blockers and 5 α -reductase inhibitors are effective, but they are expensive and have some adverse effects (Thomas, Chughtai, Kini, & Te, 2017). Although surgical treatment can remove hyperplastic prostate tissue, there are still many challenges, such as high surgical risk and poor long-term efficacy (Bhojani et al., 2014). Therefore, it is necessary and critical to investigate alternative ways

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to reduce the clinical symptoms of BPH safely and effectively.

From the traditional Chinese medicine's point of view, the key factor for the etiology and pathogenesis of BPH was the deficiency of the essence of Qi due to blood stasis. The compound of capsule of Qianlongtong has been proven to benefit or strengthen Qi for promoting blood circulation, and its clinical efficacy is significant (WenXiong Zhu, Jing Yang, ZheChun He, & Tao Liu, 2015; JiuQiao He, Wei Cai, YiFeng Yu, & Liang Zhou, 2012). However, the mechanisms underlying this are unclear. Based on our previous clinical observations, this study was designed to further explore if Qianlongtong had any direct effect on proliferation or apoptosis in hyperplastic prostate cells.

Materials and Methods

Cell Culture

Benign hyperplastic prostate cells were provided by the Cancer Research Institute of Central South University, Changsha, Hunan, China and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS). All the cells were cultured at 37°C with 5% CO₂ in a humidified incubator.

Reagents

The TUNEL and diaminobenzidine (DAB) kits were purchased from Wuhan Boster Biological Co., Ltd. Propidium iodide was provided by Shanghai Biotechnology Engineering Co., Ltd. Trizol kit was provided by Shanghai Health and Biotechnology Engineering Services Limited. β -Actin, Bcl-2, Bax, caspase-3 primers, according to the Gene Bank database query the target gene cDNA sequence application software design, synthesized by the Shanghai Biotechnology Engineering Services Co., Ltd. Reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from the Shanghai Biotechnology Engineering Co., Ltd.

Equipment

The following equipment was procured as follows: TCK3-type CO₂ constant temperature incubator, the United States Sheloln company; DK-600 electric thermostatic water tank, Shanghai Jinghong Experimental Equipment Co., Ltd.; clean bench, Suzhou Aetna air purification technology company; ECLIPSE TE300 difference inverted microscope, Japan Nikon (FACS), Coulter Corporation of the United States; PCR gene amplifiers, TC-48 / T / H (a), 96-well culture plates, 25-cm two flasks, Nuco, Japan; Taniga Geocell Imaging System (Tanon GISgel imaging system), Shanghai Tianneng

Biology Co., Ltd.; TGL16 desktop high-speed refrigerated centrifuge, Hunan Xiangyi; electrophoresis instrument, Hangzhou Dahe Co., Ltd.

Drugs

Qianlongtong capsule, composed of *Astragalus*, *Salvia miltiorrhiza*, notoginseng, pangolin, semen vaccariae, sargent-gloryvine, and other drugs, was provided by the preparation center of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine (drug approval number: 090223). To observe the toxic effect of Qianlongtong capsule liquid on the culture of prostate tissue, select 6.25 mg/L, 3.13 mg/L, 1.56 mg/L as the test drug concentration. Tamoxifen was provided by Shandong Health Pharmaceutical Co., Ltd. (drug approval number: 1210016).

Experimental Grouping

Cells were divided into control group, tamoxifen group (1.00 mg/L), high-dosage group of Qianlongtong (6.25 mg/L), moderate-dosage group of Qianlongtong (3.13 mg/L), and low-dosage group of Qianlongtong (1.56 mg/L).

The Apoptotic Index Was Detected by TUNEL Method

The apoptotic index of each prostate tissue was detected by TUNEL method. The tissue sections were fixed with 4% paraformaldehyde, paraffin embedded, and intermittently sliced; this was followed by washing, dilution, digestion, labeling, and other routine experimental steps, and finally DAB staining and hematoxylin mild restaining. On light microscopy observation, when the nucleus appears as apoptotic bodies (brown particles), those are positive cells.

$$\text{Apoptosis index (AI)} = (\text{positive cells/total number of cells}) \times 100.$$

FCM Method to Detect Cell Cycle

The cell cycle of each prostate tissue was detected by flow cytometry (FCM; Foo, 2017). The cells were treated with trypsin 1×10^6 /ml single cell suspension, followed by centrifugation, sedimentation, washing, fixation, dyeing, and other conventional experimental steps, and finally Elite ESP Analysis of flow cytometry.

The expression of Bcl-2, Bax, and caspase-3 was detected by RT-PCR. The amplified fragment length of β -actin was 442 bp; Bcl-2, amplified fragment length 330 bp; Bax, amplified fragment length 147 bp; and caspase-3, amplified fragment length 392 bp. Primer sequences used in this study are summarized in Table 1.

Table 1. Primer Sequences Used in RT-PCR.

| Name | Sense | Antisense |
|-----------|------------------------|-----------------------|
| GAPDH | TTGGCACCACACTTTCTACA | TCACGCACGATTTCCCTCTCA |
| Bcl-2 | CCATGTGTCCATCTGACC | GCCATATAGTTCCACAAA |
| Bax | CTAGCAAACCTGGTGCTCAAGG | CGAAGTAGGAGAGGAGGCCT |
| Caspase-3 | AAGCCGAAACTCTTCATCAT | CACTCCCAGTCATTCCCTTA |

Note. RT-PCR = reverse transcription-polymerase chain reaction.

Table 2. Effect of Qianlongtong on Cell Cycle of Hyperplastic Prostate Cells.

| Group | G0/G1(%) | S(%) | G2/M(%) |
|-----------------|-----------------|--------------|---------------|
| Control | 35.21 ± 2.14 | 52.37 ± 3.11 | 12.02 ± 1.09 |
| Tamoxifen | 45.32 ± 2.31** | 49.29 ± 2.35 | 5.09 ± 1.01** |
| Low dosage | 39.43 ± 1.98*## | 53.51 ± 2.12 | 6.76 ± 1.32** |
| Moderate dosage | 43.76 ± 2.05**Δ | 51.03 ± 2.29 | 5.07 ± 1.46** |
| High dosage | 47.57 ± 2.17**Δ | 49.11 ± 2.08 | 3.18 ± 0.78** |

Note: Compared with the model group, * $p < .05$, ** $p < .01$; compared with the tamoxifen group, ## $p < .01$; compared with the low-dosage group, Δ $p < .05$.

The messenger RNA (mRNA) expression levels for Bcl-2, Bax, and caspase-3 in each prostate tissue were determined by RT-PCR. The total RNA, PCR amplification, and PCR product electrophoresis were used to determine the electrophoretic density of the PCR products. The mRNA expression intensity of each gene was expressed by the ratio of electrophoretic density and β -actin (Bcl-2/ β -actin, Bax/ β -actin, caspase-3/ β -actin).

Statistical Analysis

All data were analyzed by SPSS 19.0 software. The results were expressed as mean \pm standard deviation $\bar{x} \pm s$. Multiple-group comparisons were performed using one-way analysis of variance (ANOVA). Two-group comparisons used *q*-test.

Results

Effect of Qianlongtong on Cell Cycles of Hyperplastic Prostate Cells

The control group, the tamoxifen group, and the different dosage groups of Qianlongtong were investigated in this study. Tamoxifen group was included in this study because it was shown to be effective in treating BPH by the induction of apoptosis of prostatic cells (Glienke et al., 2004). As shown in Table 2, compared with control group, the percentage of cells in G0/G1 phase increased in each group and the difference was significant ($p < .05$). Compared with low-dosage Qianlongtong group, the percentage of cells in G0/G1 phase increased in the tamoxifen group as well as in moderate- and high-dosage Qianlongtong groups and the difference was significant

($p < .01$). Compared with tamoxifen group, there was little significant difference in the percentage of cells in G0/G1 phase in high- or moderate-dosage groups ($p > .05$). On the other hand, the percentage of cells in G/M phase decreased significantly in each group when compared with control group ($p < .05$). These data suggest that Qianlongtong blocks hyperplastic prostate cells in G0/G1 phase and its effect is as significant as tamoxifen when its dosage is moderate or higher.

Effect of Qianlongtong on Apoptosis of Hyperplastic Prostate Cells

As shown in Table 3, compared with control group, the apoptotic index increased in the tamoxifen group and in Qianlongtong groups and the difference was significant ($p < .01$); compared with low-dosage Qianlongtong group, the apoptotic index increased in the tamoxifen group as well as in moderate- and high-dosage Qianlongtong groups and the difference was significant ($p < .01$). These data suggest that Qianlongtong induces apoptosis of hyperplastic prostate cells and its apoptosis-inducing effect is as significant as tamoxifen when its dosage is moderate or higher.

Effect of Qianlongtong on mRNA Expression of Bcl-2, Bax, and Caspase-3 Hyperplastic Prostate Cells

As shown in Table 4, compared with the control group, the mRNA expression of Bcl-2 was lower in the tamoxifen group as well as in the moderate- and high-dosage groups, and the difference was significant ($p < .01$ or

Table 3. Effect of Qianlongtong on Apoptosis of Hyperplastic Prostate Cells.

| Group | n | Dose (ng/ml) | Apoptosis index (%) |
|-----------------|---|--------------|---------------------|
| Control | 8 | — | 3.50 ± 0.93 |
| Tamoxifen | 8 | 1.00 | 13.38 ± 1.30** |
| Low dosage | 8 | 1.56 | 10.50 ± 2.00**# |
| Moderate dosage | 8 | 3.13 | 13.88 ± 2.03**ΔΔ |
| High dosage | 8 | 6.25 | 14.88 ± 3.64**ΔΔ |

Note. Compared with the model group, ** $p < .01$; compared with the tamoxifen group, # $p < .05$; compared with the low-dosage group, ΔΔ $p < .01$.

$p < .05$). There was a significant difference in the mRNA expression of Bcl-2 between the high-dosage group and the low-dosage group ($p < .05$).

Compared with the control group, the mRNA expression of Bax was significantly increased in the tamoxifen group as well as in the moderate- and high-dosage groups and the difference was significant ($p < .01$ or $p < .05$). There was also a significant difference in the mRNA expression of Bax between the high-dosage group and the low-dosage group ($p < .05$).

Consistent with apoptosis, compared with the control group, the mRNA expression of caspase-3 was higher in the tamoxifen group as well as in the Qianlongtong groups, and the difference was significant ($p < .01$). Compared with low-dosage group, the mRNA expression of caspase-3 was higher in moderate- or high-dosage groups ($p < .05$, $p < .01$).

Discussion

In this study, we first examined if Qianlongtong has any direct effect on proliferation or apoptosis of hyperplastic prostate cells. We found that Qianlongtong inhibits proliferation and promotes the apoptosis of hyperplastic prostate cells. The possible mechanisms by which Qianlongtong promotes apoptosis are by downregulation of Bcl-2 and upregulation of Bax.

Despite decades of study, the etiology and pathogenesis of BPH is still unclear. It might be attributed to the destruction of the balance between cell proliferation and apoptosis (Lim, 2017). This balance mechanism consists of two aspects: One is cell cycle-regulated cell proliferation, and the other is apoptosis-regulated cell death. Recent studies have shown that BPH is not only an increase in cell proliferation but also a decrease in apoptosis (Braeckman & Denis, 2017). There are dozens of genes related to apoptosis, which can be divided into three groups based on their functions (Donnell, 2011; Tang & Yang, 2009; Vignozzi et al., 2014): antiapoptotic genes, such as Bcl-2, IAP, and EIB, proapoptotic genes

such as Bax, Fas, ICE, and p53, and bidirectional regulating apoptotic genes, such as c-myc and Bcl-x. Among them, Bcl-2 and Bax are considered to be the most important regulatory genes involved in the apoptosis of prostate tissues. It is believed that many biological molecules are involved in the process of apoptosis, in which caspases are the key executors of apoptosis (Minutoli et al., 2016).

Traditional Chinese medicine has recommended that BPH be attributed to the category of “abdominal mass” (HE., Zhu, Zhang, Yuan, Zhang, & Yang, 2016). It is believed that methods to treat BPH will be effective if they could benefit or strengthen Qi by promoting blood circulation, resulting in eliminating the so-called abdominal mass (HE., 2016). Qianlongtong is a compound drug consisting of *Astragalus*, *Salvia miltiorrhiza*, notoginseng, pangolin, semen vaccariae, sargentgloryvine, and others (HE., 2016). It has been made into capsules and is widely used in the clinic. This compound drug is designed to strengthen Qi by promoting blood circulation, so that it could help eliminate the so-called abdominal mass. Our study clearly demonstrates that Qianlongtong effectively inhibits cell growth and proliferation of hyperplastic prostate cells by blocking cells in the G0/G1 cell cycle. Furthermore, our study also clearly demonstrates that Qianlongtong strongly induces the apoptosis of hyperplastic prostate cells; the possible mechanisms might be by the downregulation of antiapoptotic molecule Bcl-2 and the upregulation of proapoptotic molecule Bax. The final results favor apoptosis. This apoptosis-inducing effect has also been observed when finasteride was used in culture, although finasteride also presented an antiproliferative effect on prostatic cells (Wang et al., 2017).

In this study, we found that all dosages of Qianlongtong work well in regard to growth inhibition and induction of apoptosis. However, it should be emphasized that a high dosage of Qianlongtong seems to work better. This suggests that the effect of Qianlongtong on growth inhibition and induction of apoptosis is dose dependent. Thus, it is advised that a higher dosage is recommended to treat BPH in clinical practice. It is reasonable to argue that higher dosage might cause other problems such as side effects. However, all components in Qianlongtong are from natural traditional Chinese herbs, which are thought to have few harmful effects in the long run. Presently, a couple of drugs such as α -receptor blockers and 5 α -reductase inhibitors have been used for the treatment of BPH (Thomas et al., 2017). It has been agreed that they are effective; however, the high cost is a great burden on patients. What is more important is that both of these drugs may have severe side effects for patients. Considering effectiveness as well as safety, Qianlongtong might be a good choice for these patients.

We would like to point out the limitations of our study. The detailed mechanisms regarding why Qianlongtong

Table 4. Effect of Qianlongtong on Expression of Bcl-2, Bax, and Caspase-3 in Hyperplastic Prostate Cells.

| Group | Bcl-2 mRNA | Bax mRNA | Caspase-3 mRNA |
|-----------------|-----------------|------------------|----------------------|
| Control | 17.46 ± 3.18 | 14.12 ± 2.92 | 1.26 ± 0.03 |
| Tamoxifen | 13.35 ± 2.64** | 17.85 ± 3.51* | 1.90 ± 0.06** |
| Low dosage | 15.74 ± 4.23 | 15.43 ± 2.13 | 1.35 ± 0.04**### |
| Moderate dosage | 14.25 ± 3.86* | 17.42 ± 4.22* | 1.43 ± 0.04**###Δ |
| High dosage | 12.76 ± 3.31**Δ | 18.82 ± 3.58**ΔΔ | 1.76 ± 0.04**###ΔΔ▲▲ |

Note. Compared with the model group, * $p < .05$, ** $p < .01$; compared with the tamoxifen group, ### $p < .01$; compared with the low-dosage group, Δ $p < .05$, ΔΔ $p < .01$; compared with the moderate-dosage group, ▲▲ $p < .01$.

could block cells in G0/G1 cell need to be clarified. Many molecules might be involved in this process. Traditionally, p15, p18, p21, and p27 are regarded as antiproliferative molecules, and cyclin B, cyclin D, cyclin E, as well as cdk2 and cdk4 are regarded as pro-proliferative molecules (Fang, DeMarco, & Nicholl, 2012; Fang et al., 2007; Griffith, Brunner, Fletcher, Green, & Ferguson, 1995; Karimian, Ahmadi, & Yousefi, 2016; Minutoli et al., 2016; Ohkoshi, Yano, & Matsuda, 2015; Ohtsubo, Theodoras, Schumacher, Roberts, & Pagano, 1995; Sharma & Pledger, 2016; Sherr, 1994; Zhu et al., 2017). It will be necessary to investigate the change in these molecules caused by Qianlongtong; such a project is underway with plans to summarize the findings in a separate article. Another limitation of this study is that the effect of Qianlongtong on normal prostate and other normal cells has not been investigated. We plan to address this in the future. We realize that it might be also important to further investigate other information in patients with BPH who are taking Qianlongtong such as complete blood count (CBC) and biochemistry panels of blood to exclude potential liver or kidney damage or bone marrow inhibition. Despite these limitations, we believe that our study is important since it not only addresses the direct effect of Qianlongtong on hyperplastic prostate cells but also provides potential mechanisms by which it promotes apoptosis. This will provide a solid foundation for the clinical use of Qianlongtong.

In conclusion, Qianlongtong is a safe and effective compound to treat BPH by induction of apoptosis and inhibition of proliferation of hyperplastic prostate cells. Such a study will be helpful for clinicians to manage patients with BPH.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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