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Inhibitory Effect of Isoflavones on Prostate Cancer Cells and PTEN Gene[1]

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Objective To explore the mechanisms by which genistein and daidzein inhibit the growth of prostate cancer cells. **Methods** LNCaP and PC-3 cells were exposed to genistein and daidzein and cell viability was determined by MTT assay and cytotoxicity of the drugs by LDH test. Flow cytometry (FCM) was used to assess the cell cycle in LNCaP and PC-3 cells. Reverse transcription-polymerase chain reaction (RT-PCR) was applied to examine the expression of PTEN gene (a tumor suppressor gene), estrogen receptor alpha gene (ERα), estrogen receptor beta gene (ERβ), androgen receptor gene (AR) and vascular endothelial growth factor gene (VEGF). **Results** The viability of PC-3 and LNCaP cells decreased with increasing concentrations and exposure time of genistein and daidzein. Genistein increased G_2/M phase cells in PC-3 cells while decreased S phase cells in LNCaP cells in a dose-dependent manner. Daidzein exerted no influence on the cell cycle of LNCaP and PC-3 cells, but the apoptosis percentage of LNCaP cells was elevated significantly by daidzein. Genistein induced the expression of PTEN gene in PC-3 and LNCaP cells. Daidzein induced the expression of PTEN gene in LNCaP but not in PC-3 cells. The expression of VEGF, ERα and ERβ genes decreased and AR gene was not expressed after incubation with genistein and daidzein in PC-3 cells. In LNCaP cells, the expression of VEGF and AR gene decreased but there was no change in the expression of ERα and ERβ gene after incubation with genistein and daidzein. **Conclusion** Genistein and daidzein exert a time- and dose-dependent inhibitory effect on PC-3 and LNCaP cells. The down-regulation of ER gene by daidzein influences the growth of PC-3 cells directly. The inhibition of PC-3 cells by genistein and that of LNCaP cells by genistein and daidzein may be via Akt pathway that is repressed by PTEN gene, which subsequently down-regulates the expression of AR and VEGF genes. Our results suggest that the expression of PTEN gene plays a key role and several pathways may be involved in the suppression of prostate cancer cells by genistein and daidzein.

Key words: Isoflavones; Genistein; Daidzein; PC-3; LNCaP; PTEN; ERα; ERβ; VEGF

INTRODUCTION

Prostate cancer is one of the most common malignant carcinomas in Western countries, with a high morbidity and mortality. It is the second leading cause of cancer death after lung cancer in men[1]. Furthermore, there are no effective therapies after the conventional treatment failure, so it is very important to find new preventive and therapeutic measures. In recent years, epidemiological researches found that the morbidity of prostate cancer in Asia where people ingest more bean products is significantly lower than that in the Western countries[2]. Moreover, studies discovered that the morbidity of prostate cancer is negatively correlated with the intake of isoflavones[3-4]. It has been demonstrated that isoflavones inhibit the growth of prostate cancer cells both in animals and *in vitro*. Isoflavones, abundant in soybeans, are composed of three active components: genistein, daidzein and glycitein. Their structures are similar to 17β- estrogen[5].

In the field of prostate cancer research, much attention has been focused on PTEN gene (phosphatase and tensin homology deleted on chromosome ten, also known as MMAC1/TEP). Mapped to chromosome 10q23.3, PTEN gene is a tumor suppressor gene discovered recently. PTEN dephosphorylates phosphatidylinositol (3, 4, 5)-triphosphate (PtdIns-3, 4, 5-P3), an important intracellular second messenger, and reduces its level within the cell. By dephosphorylating PtdIns-3, 4, 5-P3, PTEN acts in opposition to phosphatidylinositol 3-kinase (PI3K), which has a pivotal role in the production of PtdIns-3, 4, 5-P3. The latter is

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necessary for the activation of Akt, a serine/threonine kinase involved in cell growth and survival. By blocking the activation of Akt, PTEN regulates cell activities such as cell cycle, translation, and apoptosis. PTEN is inactivated frequently in prostate cancer, leading to apoptosis suppression. At present, PTEN and the Akt pathway are thought to play key roles in the prevention and therapy of prostate cancer[6].

The function of estrogen receptor (ER) in prostate cancer, a hormone-related tumor, has been recognized in recent years. ERs have different subtypes, mainly including ERα and ERβ[7]. They share a high amino acid homology (95% DNA binding domain; and 55% ligand binding domain), and ERα stimulates transcription and cell proliferation. Both of them may be involved in the development and progression of prostate cancer[8].

In prostate cancer, one of the most powerful stimulators of angiogenesis is the vascular endothelial growth factor (VEGF). VEGF transcription could be induced by hypoxia through the activation of the PI3 kinase pathway[9]. VEGF has four isoforms, VEGF121, VEGF165, VEGF189, and VEGF206[10]. In addition, androgen receptor (AR) is a key transcription factor in prostate cancer. The expression, activation and up-regulation of AR gene may play a pivotal role in prostate cancer[11].

In order to explore the mechanisms of genistein and daidzein on prostate cancer cells, we studied the viability, cell cycle changes of prostate cancer cells treated with genistein and daidzein respectively. Furthermore, we investigated the expression of PTEN, ER, AR and VEGF genes in prostate cancer cells before and after genistein and daidzein treatment.

MATERIALS AND METHODS

Reagents and Cell Lines

Androgen dependent LNCaP cells and androgen independent PC-3 cells were obtained from Shanghai Institute of Cell Biology[12], Chinese Academy of Sciences. All cells were maintained as monolayer in a humidified atmosphere of 5% CO_2 in air in F-12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). MTT (3-[4, 5-dimethylthiazol-2-yl]-2-, 5-diphenyltetrazolium bromide, 5 mg/mL), genistein and daidzein were purchased from Sigma Scientific (St Louis, MO, USA). Genistein and daidzein were made into 0.1 mol/L stocking solution in DMSO and the stocking solution was diluted into 10, 20, 40, 60, 80, and 160 μmol/L. Frozen stocks of each cell line were established and used.

Growth Inhibition of Prostate Cancer Cells by Phytochemicals

PC-3 and LNCaP cells were seeded at a density of 5×10^3 cells/well in 96-well plates (CORNING), and the test reagents were added. Controls were treated with same amount of DMSO. All experiments were performed in triplicate.

MTT-assay After incubation for 24 hours (PC-3 cells) and 48 hours (LNCaP cells), a series of five concentrations of genistein and daidzein (10, 20, 40, 80, and 160 μmol/L, respectively) were added. At 24, 48, and 72 hours, 20 μL of the supernatant from each well was gently aspirated into microcentrifuge tubes and stored at -37℃ until assayed for lactate dehydrogenase (LDH) activity. At each time point, the adherent cells in each well were lysed and solubilized for MTT assay[13]. The absorbance was read on a universal microplate reader (BIO-TEK, EL ×800) at 490 nm and 630 nm. The relative numbers of live cells was determined based on the optical absorbance (optical density, OD). We calculated the viable cells according to the following equation:

$$\% \text{ viable cells} = (T_{OD} - B_{OD})/(C_{OD} - B_{OD}) \times 100$$

where T_{OD} is the absorbance of the treated cells (mean value), C_{OD} is the absorbance of the control (mean value), B_{OD} is the absorbance of the blank (mean value)

LDH-cytotoxicity test To determine the cytotoxicity of genistein and daidzein to PC-3 and LNCaP cells, LDH activity in the previously frozen supernatants was determined at 24 and 48. Briefly, 20 μL supernatant from each well was transferred into correspondingly labeled tubes, and 500 μL basic buffer and 100 μL trihydrate (NAD, AMRESCO) were added to each tube. The tubes were incubated for 15 min at 37℃, then 500 μL 2, 4-dinitrophenylhydrazine was added, and incubated for 15 min at 37℃ again. Finally, 5 mL 0.4 mol/L NaOH was added, and the absorbance was measured at 440 nm on spectrophotometer number 721, and the activity of LDH was calculated according to the standard curve. All tests were performed in triplicate. This assay was based on the release of the cytoplasmic enzyme LDH from cells. Dying cells experienced extensive breakdown of the cellular membrane system and release of LDH. Thus, in cell culture, the course of cytotoxicity could be followed quantitatively by measuring the activity of the cytoplasmic enzyme LDH in the supernatant.

Cell Cycle Analysis

PC-3 and LNCaP cells were incubated with genistein and daidzein, and harvested by 0.25% trypsin after 48 h. Cell cycle analysis was performed

using the method of Crissman[14]. Samples were analyzed using a flow cytometer (EPICS VI, PE Inc.). All tests were performed in triplicate.

RNA Extraction and RT-PCR

Total RNA extract was prepared using Trizol reagent (Invitrogen Technologies) according to the manufacturer's instructions. Single-stranded cDNA was prepared using AMV reverse transcriptase (Bio Basic Inc.) with Oligo-T^{18} primer. The cDNA was subjected to PCR reaction using human PTEN, AR, ERα, ERβ and VEGF gene specific primers, and the PCR reactions used ready-to-use PCR kit (Sangon Inc. Canada). The control housekeeping gene (β-actin gene) was also amplified under the same conditions for normalization of PTEN[15], AR, ERα, ERβ[16] and VEGF expression. VEGF primers were designed for amplification of the four isoforms (VEGF121, VEGF165, VEGF189, and VEGF206) of VEGF[17]. The primers used in the PCR reaction are presented in Table 1. Each cycle consisted of denaturing for 30 s at 94℃, annealing for 30 s at 55℃, and polymerization for 2 min at 72℃, and the reactions were processed in PCR instrument (PEKIN ELMER, GeneAmp 9600). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The optical densities (OD) of PCR products in agarose gel were scanned and measured with a densitometer (Gel Doc 2000, BIO-RAD), and quantified using molecular analyst software (Bio-Rad). The ratio of each specific gene against β-actin was calculated by standardizing the ratio of each control.

Statistical Analysis

All experiments were carried out in triplicate and repeated twice. Significance of the differences in mean values was determined using the one-way ANOVA, and $P<0.05$ was considered statistically significant.

TABLE 1

Primers Used for RT-PCR Analysis

Genes	Primer Sequence	PCR Product (bp)
PTEN	ACAGCCATCATCAAAGAGATCG	750
	CACCACACACAGGTATCGGC	
Androgen Receptor	CCGTCCAAGACCTACCGA	248
	GGAGAACCATCCTCACCCT	
Estrogen Receptorα	GATGTGGGAGAGGATGAGGA	153
	AGCACCCTGAAGTCTCTGGA	
Estrogen Receptorβ	TTC CCA GCA ATG TCA CTA ACT	259
	CTC TTT GAA CCT GGA CCA GTA	
VEGF[18]	ATGAACTTTCTGCTGTCTTGGG	VEGF121: 514
	CTGTATCAGTCTTTCCTGGTGAG	VEGF165: 646
		VEGF189: 718
		VEGF206: 769

RESULTS

Genistein and Daidzein Inhibited Growth and Proliferation of PC-3 and LNCaP Cells

Genistein and daidzein inhibited growth and proliferation of PC-3 cells MTT assays revealed that the viability of PC-3 cells decreased with the increasing concentration and treatment time of genistein and daidzein. At 72 h, the viability of PC-3 cells decreased to 12.28% after genistein treatment (160 μmol/L) and 44.88% after daidzein treatment (160 μmol/L). Genistein over 10 μmol/L and daidzein over 40 μmol/L inhibited the proliferation of PC-3 cells (Fig. 1). PC-3 cells were damaged only at 48 h post-treatment and released the cytoplasmic dehydrogenase after genistein and daidzein treatment at 160 μmol/L. LDH levels significantly increased to 1091 U/L ($P<0.01$) and 827 U/L ($P<0.05$), respectively.

Genistein and daidzein inhibited growth and proliferation of LNCaP cells Growth and proliferation of LNCaP cells were inhibited by genistein and daidzein in a does- and time-dependent manner (Fig. 2). At 72 h, the viability of LNCaP cells decreased to 25.48% after genistein treatment and 43.14% after daidzein treatment at the dose of 160 μmol/L each. Genistein at the concentration over 20 μmol/L and daidzein over 10 μmol/L inhibited the growth of LNCaP cells ($P<0.01$). The increased level of LDH released by LNCaP cells indicated that LNCaP cells were damaged by genistein and daidzein at 160 μmol/L (both $P<0.05$).

Cell Cycle Dynamics

After incubation for 48 h with genistein and

daidzein, the changes of cell cycle of both cell lines were investigated. Genistein increased G_2/M phase cells dose-dependently in PC-3 cells[18], and enhanced DNA content in G_2/M phase to 14.88%, 30.56% at 20 and 60 μmol/L, respectively (P<0.05) (Table 2). However, no distinct changes in PC-3 cells were found after daidzein treatment. In LNCaP cells, genistein not only increased G_2/M phase but also decreased S phase cells dose-dependently, with a significant difference compared with controls (P<0.05)[19]. Although daidzein exerted no influence on the phases of LNCaP cell cycle, the percentages of apoptotic cells were significantly elevated, which were 18.39% (P<0.05) and 27.64% (P<0.01) at 20 and 60 μmol/L, respectively. It demonstrated a dose-response relationship between daidzein and apoptosis rate of LNCaP cells.

Regulation of mRNA Expression by Genistein And Daidzein

The expression of PTEN gene was up-regulated in PC-3 cells after genistein treatment, however there was no detectable expression of PTEN gene after daidzein treatment. The expressions of ERα and ERβ gene were down-regulated in PC-3 cells after treatment with genistein and daidzein (Fig. 3).

Compared with the controls, the expression of PTEN, ERα and ERβ gene after genistein and daidzein treatment was significantly different in PC-3 cells (P<0.05). VEGF isoforms, 121 and 165 (from up to down), were detected in controls, but isoforms 189 and 206 were absent in PC-3 cells. VEGF 121 was the most abundant and therefore was considered to be the major isoform in PC-3 cells. The expression of VEGF gene was distinctly down-regulated by genistein in PC-3 cells, and showed no alterations after daidzein treatment. After incubation with genistein and daidzein, the expression of AR gene was not detected in PC-3 cells.

The expression of PTEN gene in LNCap cells was up-regulated after genistein and daidzein treatment, and gradually increased with increasing dose of genistein, showing a dose-response relationship. The expression of AR gene was down-regulated after genistein and daidzein treatment. VEGF isoforms 121, 165 and 189 (from

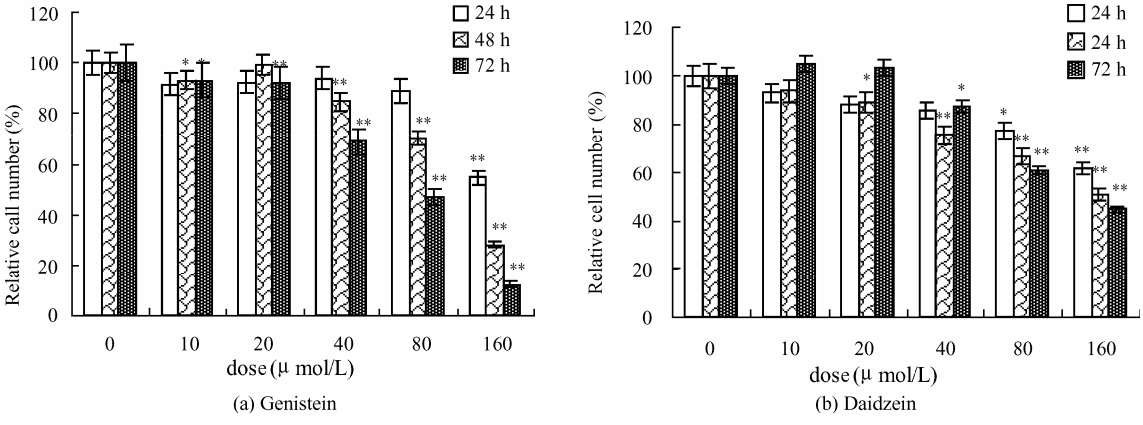

FIG. 1. Viability of PC-3 cells treated with genistein and daidzein. $^{*}P$<0.05; $^{**}P$<0.01.

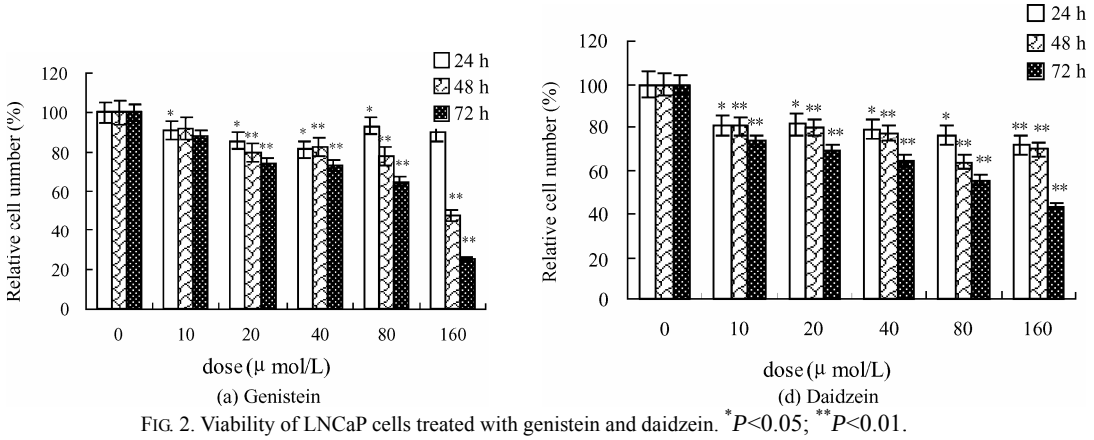

FIG. 2. Viability of LNCaP cells treated with genistein and daidzein. $^{*}P$<0.05; $^{**}P$<0.01.

TABLE 2
Changes of Cell Cycle of PC-3 Cells After Genistein and Daidzein Treatment ($\bar{x} \pm s$)

	Control	Genistein (μmol/L)		Daidzein (μmol/L)	
		20	60	40	80
G_0+G_1 (%)	55.85±7.5	48.11±6.6	49.35±3.4	52.46±5.7	51.67±6.6
S (%)	33.93±2.3	37.01±8.2	20.28±3.1	36.52±8.4	40.96±11.1
G_2+M (%)	10.22±3.2	14.88±6.6*	30.56±2.2**	11.02±1.0	7.36±1.3
Apoptosis (%)	3.9±1.2	11.75±2.3	13.54±3.2	9.39±1.3	12.54±2.4

Note. * $P<0.05$; ** $P<0.01$.

TABLE 3
Changes of Cell Cycle of LNCaP Cells After Genistein and Daidzein Treatment ($\bar{x} \pm s$)

	Control	Genistein (μmol/L)		Daidzein (μmol/L)	
		20	60	20	60
G_0+G_1 (%)	55.30±6.0	26.34±3.78	56.38±3.03	52.99±1.98	60.50±3.29
S (%)	31.07±4.6	20.84±1.73*	13.11±3.33**	34.77±3.19	26.82±6.17
G_2+M (%)	13.63±1.7	20.21±0.93*	30.51±2.19**	12.24±1.54	12.67±2.98
Apoptosis (%)	10.06±1.3	6.14±1.04	7.57±1.69	18.39±0.74*	27.64±7.88**

Note. * $P<0.05$; ** $P<0.01$.

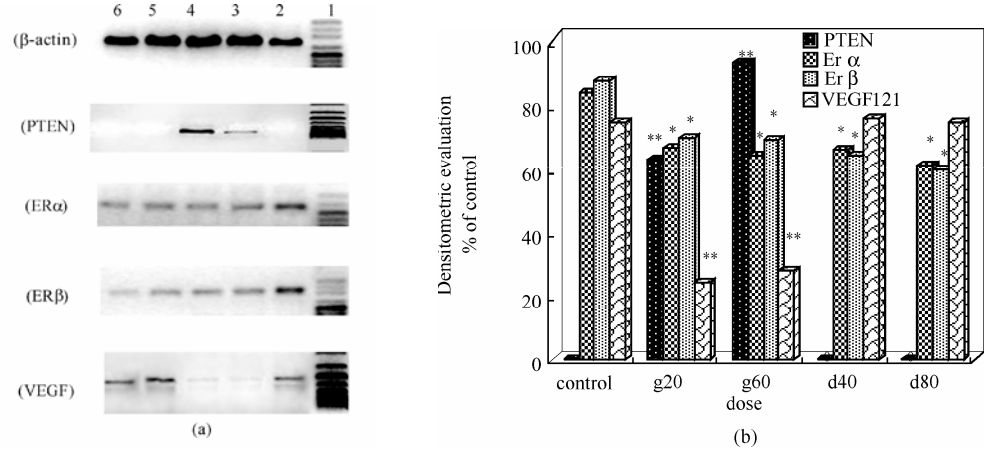

FIG. 3. RT-PCR analysis of PTEN, ERα, ERβ, and VEGF genes in LNCaP cells treated with genistein and daidzein. (a) 1, marker; 2, control cells; 3, genistein 20 μmol/L; 4, genistein 60 μmol/L; 5, daidzein 20 μmol/L; 6, daidzein 60 μmol/L. (b) *$P<0.05$; **$P<0.01$.

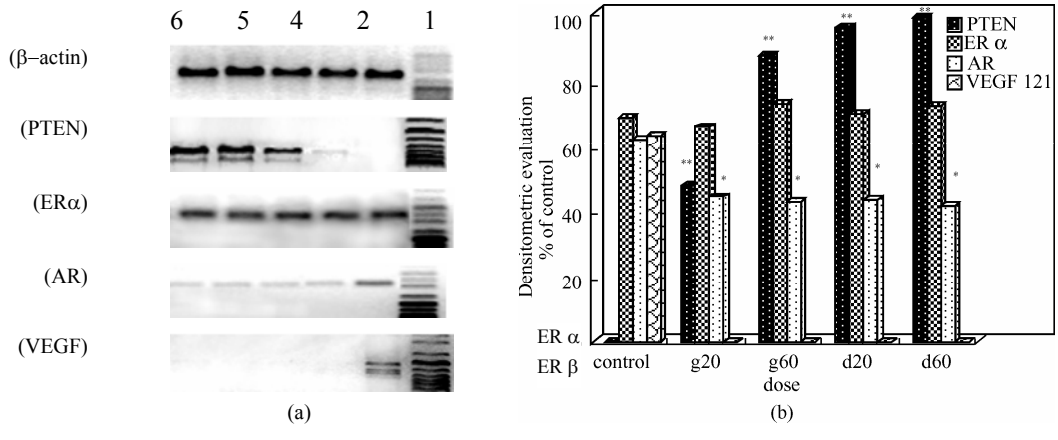

FIG. 4. RT-PCR analysis of PTEN, ERα, AR, and VEGF genes in LNCaP cells treated with genistein and daidzein. (a) 1, marker; 2, control cells; 3, genistein 20 μmol/L; 4, genistein 60 μmol/L; 5, daidzein 20 μmol/L; 6, daidzein 60 μmol/L. (b) *$P<0.05$; **$P<0.01$.

up to down) were detected in controls, but isoform 206 was absent in LNCaP cells. VEGF 121 was the most abundant isoform in LNCaP cells. After incubation with genistein and daidzein, the expression of VEGF gene was down-regulated, and no expression was found in treated LNCaP cells (Fig. 4).

DISCUSSION

Since soybean consumption is one of the principal factors for prevention of prostate cancer, more attention has been paid to phytoestrogens. Genistein and daidzein, as the major isoflavonoids presented in soybeans, can inhibit the growth of prostate cancer in animals and *in vitro*, but the underlying mechanisms are unclear. In the present study, we investigated the changes of cell viability, cell cycle and the expressions of PTEN, AR, ER and VEGF genes in PC-3 and LNCaP cells after incubation with genistein and daidzein.

The results of MTT and LDH assays indicate that genistein and daidzein could damage the cell membrane at high doses (160 μmol/L). With increasing doses and longer time of treatment, the viability of both cell lines decreases[20].

It was demonstrated that genistein could not only arrest cells at G_2/M phase in both cell lines but also interrupt the synthesis of DNA in LNCaP cells. The apoptosis rates of PC-3 and LNCaP cells elevated with increasing doses of genistein, but without significant differences compared with the control group. A dose-dependent apoptosis induced by daidzein was shown in LNCaP cells.

To further elucidate the molecular mechanisms of genistein and daidzein underlying the suppression of cell growth in both cell lines, we investigated the expression of PTEN, ERα, ERβ, AR and VEGF genes. PTEN, a lipid phosphatase, regulates the Akt pathway, an important target pathway for prevention and therapy of prostate cancer. A number of studies have demonstrated that after PTEN-negative PC-3 and LNCaP cells are transfected with a PTEN expression plasmid, the transfected PC-3 and LNCaP cells show higher responses to the cytotoxic agent[15,21]. Our results indicate the PTEN gene is not expressed in untreated PC-3 and LNCaP cells[15]. After incubation with genistein and daidzein, PTEN gene was expressed except in PC-3 cells treated with daidzein. The inactivation of PTEN occurs at the level of transcription in prostate cancer[22]. So isoflavones may influence the transcription of PTEN gene.

The expression of ERα and ERβ gene in PC-3 cells decreased after genistein and daidzein treatment, indicating that isoflavones influence the pathway of estrogen receptor[23]. The results are consistent with previous studies[24]. The possible mechanisms are that isoflavones acting as a ligand and ER partial agonist/antagonists interact with ERs[25]. In previous studies, administration of isoflavones to castrated mice has been shown to up-regulate the estrogen-responsive genes in the prostate, further suggesting an estrogenic mechanism. Estrogenic compounds administered to intact neonatal rats permanently up-regulate ERα expression in the prostate[26-27]. However, prostate ERα and ERβ were down-regulated after incubation with genistein and daidzein in our study. This difference may be due to the lower estrogenic activity of genistein and daidzein compared to endogenous estrogens[28]. Typically, estrogenic responses in males have been observed utilizing high doses of genistein and daidzein[29]. Also, neonatal estrogen treatment reduces prostate size and androgen sensitivity in the androgen deprived environment, which may result in a greater proportion of ER positive cells interpreted as ER up-regulation.

In PC-3 cells, the expression of VEGF gene decreased after incubation with genistein, but there were no obvious changes after daidzein treatment. It might be due to the changes of Akt pathway. The results indicate that genistein up-regulates the expression of PTEN gene in PC-3 cells, then dephosphorylates D3 position of PtdIns-3, 4, 5-P3, lowering its level within the cells. This action affects many different cellular processes but its effect on Akt is the most scrutinized. By dephosphorylating PtdIns-3, 4, 5-P3, PTEN acts in opposition to PI3K, thus inhibiting the activation of Akt. It further decreases the expression of VEGF gene, a downstream target gene in Akt pathway. This suggests a possible role of genistein in angiogenesis. On the contrary, PC-3 cells after daidzein treatment do not express PTEN gene, and the expression of VEGF gene has no distinct change. The consistent results suggest that daidzein does not influence the pathway of Akt. Therefore, the mechanisms of genistein and daidzein might be different in PC-3 cells, and genistein might influence both the pathways of Akt and ERs. Although daidzein does not influence the pathway of Akt, PC-3 cells are also inhibited by daidzein. The expression of ERs decreases after incubation with daidzein. The results of MTT have shown that the lowest available concentrations and LC_{50} in PC-3 cells after genistien treatment are lower than those after daidzein treatment (10 μmol/L vs 40 μmol/L and 70 μmol/L vs 110 μmol/L), thus further supporting the above-suggested

mechanisms.

In LNCaP cells, the expression of ERα gene had no distinct change, but the expression of VEGF gene was significantly down-regulated after incubation with genistein and daidzein. This result is in conformity with the up-regulation of PTEN gene induced by genistein and daidzein treatment. The inhibition of growth by genistein and daidzein may be through the pathway of Akt in LNCaP cells. Some recent studies found that there is a cross-talk between the pathways of Akt and androgen[30]. PTEN negatively regulates the Akt pathway and represses AR activity. Akt elevates AR activity to stimulate prostate cell growth and prolong survival. Based on the current study we proposed that Akt activity decreased after genistein and daidzein treatment in LNCaP cells, and the expression of AR was down-egulated via the cross-talk between Akt pathway and AR pathway[31]. In addition, some previous studies showed that ERβ regulates the expression of AR gene in LNCaP cells, but in our study, there was no expression of ERβ gene in LNCaP cells[32].

In conclusion, genistein and daidzein exert time- and dose-dependent inhibitory effects on the growth and proliferation of PC-3 and LNCaP cells, and play a critical role in inhibiting prostate cancer cells.

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