Int J Clin Exp Pathol 2018;11(4):2025-2031
www.ijcep.com /ISSN:1936-2625/IJCEP0059707

Original Article

The role of fenhexamid on the proliferation of ovarian cancer BG-1 cells

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Received September 24, 2016; Accepted August 16, 2017; Epub April 1, 2018; Published April 15, 2018

Abstract: To study the influence of fenhexamid in pesticide residue to the human ovarian cancer BG-1 cell proliferation. Detecting the effectiveness of 17β-estradiol, fenhexamid and Fulvestrant to BG-1 cell proliferation by MTT, and detecting the expression levels of cyclin D1 and cyclin E by Western blot. Fenhexamid can promote BG-1 cell proliferation for its estrogen-like effect. On the other hand, it can help to improve the expression levels of cyclin D1 and cyclin E in BG-1 cells which is regulated by ER-dependent pathway. And 17β-estradiol is also regulated by the same way. The existence of fenhexamid can promote ovarian cancer cell proliferation, so for patients with ovarian cancer, fenhexamid in pesticide residue may make medical conditions worse.

Keywords: Ovarian cancer, fenhexamid, pesticide residues

Introduction

Ovarian cancer is one of the common malignant tumors in female reproductive system which seriously threats women's life and health. In recent years, scientific research workers' study about the exogenous substances in the environment which can cause cancer has been a hot area of research. Endocrine-disrupting chemicals (EDCs), also called environmental hormone, is a kind of chemical substance [1] which can interfere with the body endocrine system and at the same time, it can cause abnormal effects by working with the relative receptors. EDCs are similar to estrogen or androgen structure which can competitively combining with the relative receptors. So EDCs can interfere with endogenous steroids, disturbing the balance of the endocrine and vivo system [2]. Furthermore, these compounds can inhibit endogenous hormones by helping to change the three-dimensional conformation of estrogen receptor (ER). Then they can influence the expression of coenzyme factor [3]. The interactions of EDCs and ERs control the committed step of ER-mediated gene transcription regulation and regulate the expression of ER target genes [4]. And estrogen (E2) which can regulate the development of reproductive system can also activate the reproductive organs such as mammary gland, ovary, endometrium and so on. They are all the expressions of ERs [5]. The excessive expression of E2 or the stimulation of exogenous compounds such as bisphenol A, phenol and benzophenone which is similar to E2 can induce the occurrence of estrogen-sensitive cancers of the reproductive organs [6-8].

The proliferation of normal cells is regulated by cell cycle and every factor in each phrase of cell cycle can be the checking point [9] such as cyclin D1, p21, p27 and cyclin E. These proteins are closely linked with cell cycle progression plays a key role in cell proliferation. But for cancer cells, there genes who regulate the cell cycle has been unbalanced. For example, cyclin D1 and p2 are stimulated by EDCs such as bisphenol A or octyl phenol and will be overexpressed [10, 11] by ER pathway in ovarian cancer. More than that, benzophenone can stimulate the growth of BG-1 ovarian cancer

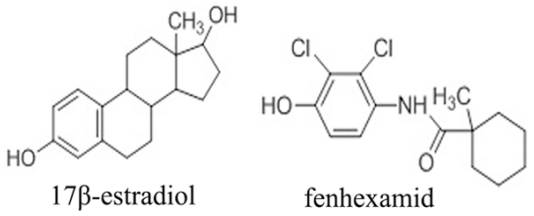

Figure 1. Structures of 17β-estradiol (E2) and fenhexamid.

cells [6] by the same way. These results indicate that the unbalance of checking points of cell cycle which is caused by ECDs can make cancer over proliferation by ER-dependent pathway.

Pesticides are widely applied in pest control to ensure agricultural production [12]. Fenhexamid (2',3'-dichloro-4'-hydroxy-1-methylcyclohexane formanilide) can restrain C3-keto reductase to influence the biosynthesis of ergosterol. This compound had been widely used to be foliage fungicide since 2000 and is a kind of hydroxy amine compound. It mainly controls gray mold and is widely applied to some agricultural production such as grapes, stone fruits, citrus fruits, vegetables, strawberries, blackcurrants, sweet cherries and so on. So many fruits from the harvest can be detected to have fenhexamid though its level is lower than the statutory maximum residue levels [13]. In addition, the residual fenhexamid is often detected in comprehensive program of pesticide residues' detection, it even can be found in bottled wines which are in the sale [14]. Studies have reported that in MCF-7 breast cancer cells, fenhexamid can alter ER target genes such as the expressions of cyclin D1 codon, progesterone receptor and nuclear respiratory factor 1 [15].

Though there are some potential risks that pesticides help to promote the presence of various diseases, there are no relevant reports about the specific effects of pesticides on the human body until now. So this research's aim is to study the influence of fenhexamid, the common pesticide, to the expressions of estrogen-sensitive human ovarian cancer BG-1 cell proliferation, cyclin D1 and cyclin E.

Materials and methods

Cell strain

Human ovarian cancer cells BG-1 were purchased from Shanghai Institute of Biochemistry and Cell Biology Life Sciences in China.

Reagents and drugs

Dulbecco's modified Eagle's medium (DMEM) culture medium, phenol red-free DMEM medium, fetal calf serum, fetal bovine serum which is treated by charcoal or dextran were all purchased from Gibco Company in America; MTT, 17β-estradiol (E2, an estrogen), fulvestrant (ICI 182780, an estrogen receptor antagonist), fenhexamid and cell lysis protein extraction kit were all purchased from Sigma Company in America (structures of E2 and fenhexamid were showed in **Figure 1**); polyvinylidene fluoride membrane (PVDF) was purchased from Millipore Company in America; the primary antibodies are mouse anti-cyclin D1, cyclin E (1:3000 dilution, Abcam Company in UK) and GAPDH (1:10,000 dilution, Abcam Company in UK); the secondary antibody is horseradish peroxidase-labeled anti-mouse antibody which was purchased from Santa Cruz Biotechnolog Company; enhanced chemiluminescence (ECL) detection reagents were purchased from Pierce Company in America; and the other reagents were analytical grades which were made in China.

Cell culture

The BG-1 cells were cultured in DMEM medium which contained 10% fetal bovine serum. Then it was cultured in an incubator which contained 37°C and 5% saturated moist CO_2. Logarithmic growth phase cells would be used for planking in the experiment. To avoid the estrogen effect in the ingredients of DMEM and fetal calf serum, the DMEM medium in the experiment would contain 5% fetal bovine serum without phenol red which was treated by charcoal or dextran.

Detected the proliferation of BG-1 cells by MTT method

BG-1 cells which were in the logarithmic growth phase were diluted to cell concentration of 5×103 cells per well and then they were seeded in 96-well plates. After two days, it would be replaced to the experimental medium to take administered test. There would be seven groups in this experiment. They were the blank group (without nutrient solution of cell experiment), the control group (with nutrient solution of cell experiment), ICI (10^{-8} M) negative control group, E2 (10^{-9} M) positive control group, administration group of E2 (10^{-9} M) and ICI (10^{-8} M), fenhexamid (10^{-5}-10^{-8} M) administration

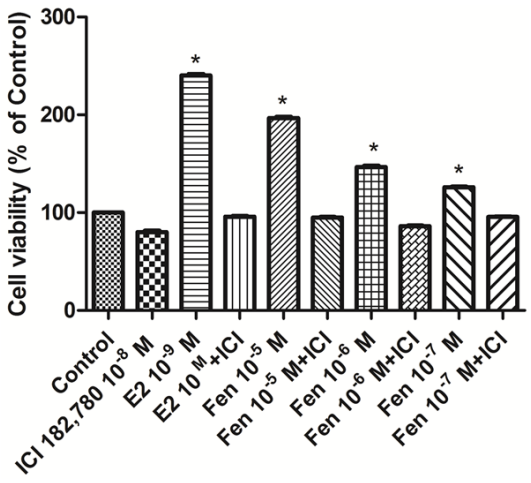

Figure 2. Increased cell proliferation of BG-1 cells following treatment with E2 and fenhexamid (Fen). Cells were divided into control, E2 (10^{-9} M) and fenhexamid (10^{-5}-10^{-7} M) groups in the presence or absence of ICI 182,780 (10^{-8} M) for 9 days. Values are the mean ± standard deviation (S.D.). *Mean values were significantly different from those for the control, $P < 0.05$.

group and administration group of fenhexamid (10^{-5}-10^{-8} M) and E2 (10^{-9} M). There would be 6 wells in each administered concentration and be replaced per three days. After nine days, the original culture medium would be sucked out. After replaced the fresh experimental medium, MTT (5 mg/ml) 20 ul/well would be added. After four hours, we sucked out the liquid in the wells and added DMSO 150 ul/well. After shaking it at a low speed for ten minutes, each well's absorbance was measured at 570 nm. Time was used to be the abscissa, OD value was used to be the vertical and then we would calculate the cell survival rate. Cell survival rate = (OD value in administration group - OD value in blank control group)/(OD value in control group - OD value in blank group)*100%. The experiment would be repeated three times.

Detected the expression levels of Cyclin D1 and Cyclin E by Western blot

BG-1 cells which were in the logarithmic growth phase were diluted to cell concentration of 1×105 cells per well and then they were seeded in 6-well plates. After two days, it would be replaced to the experimental medium to take administered test. There would be six groups in this experiment. They were the control group (with nutrient solution of cell experiment), ICI (10^{-8} M) negative control group, E2 (10^{-9} M) positive control group, administration group of E2 (10^{-9} M) and ICI (10^{-8} M), fenhexamid (10^{-5} M) administration group and administration group of fenhexamid (10^{-5} M) and E2 (10^{-8} M). We would wait for 24 hours, 48 and 72 hours after administration in each group, then extract its total cellular protein respectively and measure its concentration by BCA method. Total protein was separated by using 12% SDS-PAGE electrophoresis and transferred to a PVDF membrane. Then it was closed by BSA solution at room temperature for one hour. Primary antibodies were hatched overnight at 4°C. After PVDF membrane was washed by TBST solution 3 times and every time is five minutes, the secondary antibodies were hatched for one hour at room temperature. After the membrane was washed by TBST solution 3 times and every time lasted 5 minutes, we would dropwise add ECL luminescent reagent to make the color. Then it was pressed by X tablet to exposure imaging.

Data analysis

The data was analyzed by using GraphPad Prism software. Experimental data was presented as mean ± SD, and the mean was compared by using one-way ANOVA. There was statistically significant when $P < 0.05$.

Results

The influence of fenhexamid in the proliferation of BG-1 ovarian cancer cells

The results of MTT experiment suggested that compared with the control group, ICI negative control group has some anti-proliferative activities. But when E2 and fenhexamid individually acted on BG-1 cells, its cell proliferation activity was significantly increased. Under the action of E2 10^{-9} M, there were significant differences in the proliferation activity of BG-1 cells when it was compared with the control group ($P < 0.05$). When the concentration of fenhexamid was at 10^{-8} M or below it, there was no influence in the BG-1 cells proliferation. But when the concentration of fenhexamid was over 10^{-7} M, there were significant differences in the proliferation activity of BG-1 cells when it was compared with the control group ($P < 0.05$). And the effect on the proliferation of BG-1 cells was gradually enhanced with the increase of fenhexamid's concentration. So it exhibited the concentra-

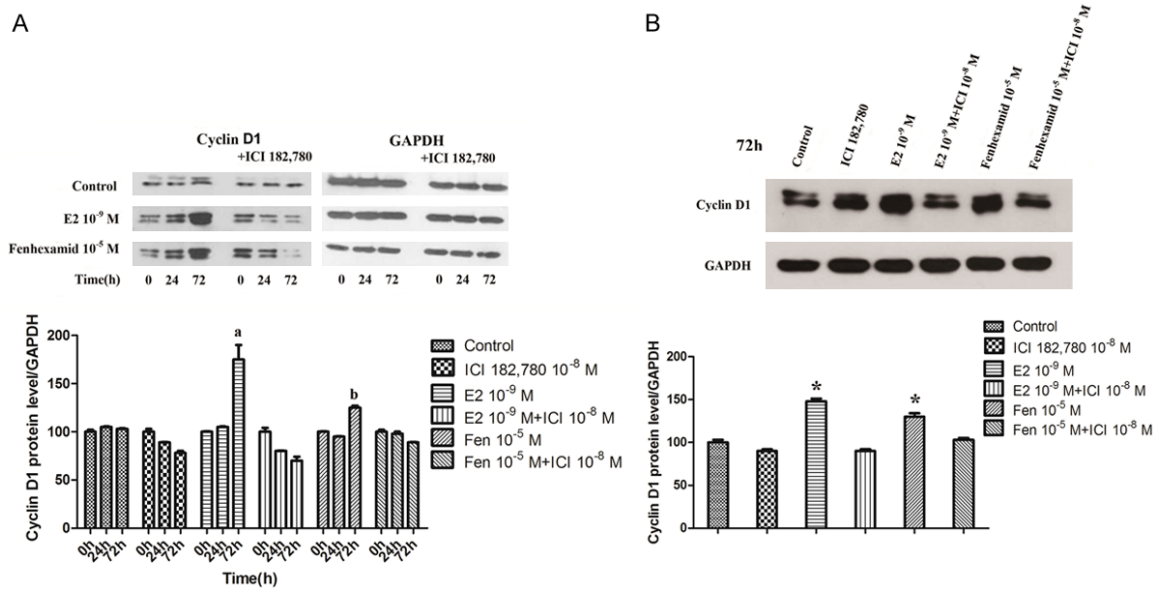

Figure 3. Altered protein expression of cyclin D1 following treatment with E2 and fenhexamid (Fen). BG-1 cells were seeded in six well plates and divided into control, E2 (10^{-9} M) and fenhexamid (10^{-5} M) in the presence or absence of ICI 182,780 (10^{-8} M) for 24 and 72 h (A) or 72 h (B). a Mean values were significantly different from those for 0 h of E2 (10^{-9} M) incubation, $P < 0.05$. b Mean values were significantly different from those for 0 h of fenhexamid (10^{-5} M) incubation, $P < 0.05$. *Mean values were significantly different from those for the control, $P < 0.05$.

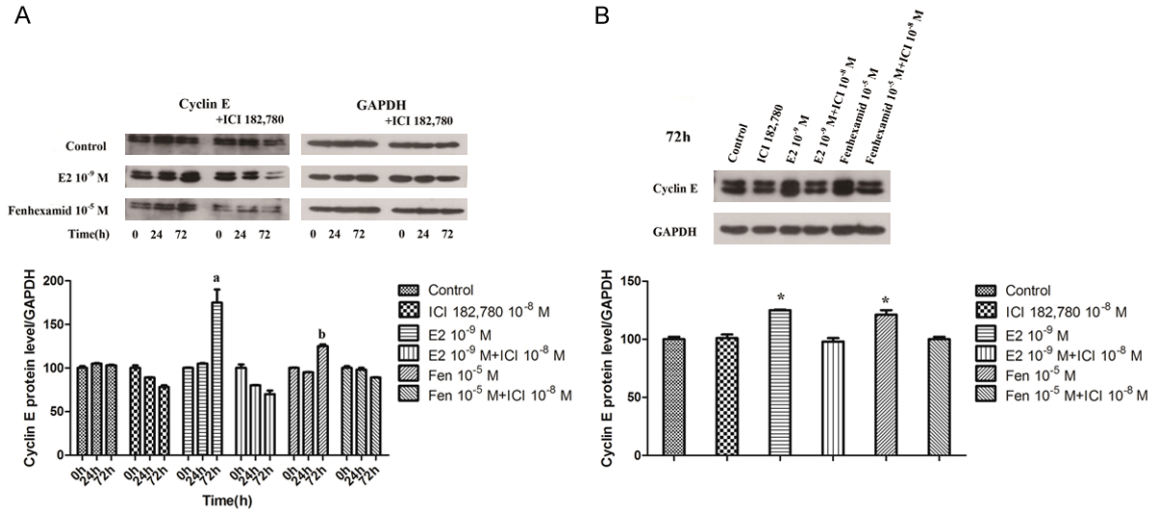

Figure 4. Altered cyclin E protein expression following treatment with E2 and fenhexamid (Fen). BG-1 cells were seeded in six well plates and divided into control, E2 (10^{-9} M) and fenhexamid (10^{-5} M) in the presence or absence of ICI 182,780 (10^{-8} M) for 24 and 72 h (A) or 72 h (B). a Mean values were significantly different from those for 0 h of E2 (10^{-9} M) incubation, $P < 0.05$. b Mean values were significantly different from those for 0 h of fenhexamid (10^{-5} M) incubation, $P < 0.05$. *Mean values were significantly different from those for the control, $P < 0.05$.

tion-dependence. And at the concentration of 10^{-5} M, the effect on the proliferation of BG-1 cells was the same as the effect on the proliferation of E2 cells (see **Figure 2**). The experiment results of E2 or fenhexamid which was administered in combination with ICI showed that the effect on the proliferation of cells was obviously decreased, and there were no significant differences in the proliferation activity when it was compared with the control group (see **Figure 2**). The results showed that when estrogen receptor antagonist ICI existed, the

effect on the proliferation of E2 and fenhexamid were all suppressed.

The influence of fenhexamid on the expression of Cyclin D1

According to the results of MTT experiment, and for the effect of fenhexamid on BG-1 cells was more significantly observed, fenhexamid was worked on the BG-1 cells at the concentration of 10^{-5} M in the Western blot experiment. The results of Western blot experiment suggested that after 24 hours and 48 hours when fenhexamid and E2 were worked on the BG-1 cells, the expression level of Cyclin D1 was significant increased when it was compared with the Cyclin D1 of the control group. There was significant difference ($P < 0.05$). It acted the time-dependent manner. The expression level of Cyclin D1 was enhanced with the increase of the time after drug treatment (see **Figure 3A**). There was no significant changes when the expression level of Cyclin D1 was compared with CycIn D1 of the control group under the situation that after 24 hours and 48 hours when fenhexamid and E2 were worked on the BG-1 cells in the combination with ICI (10^{-8}) (see **Figure 3A**). The expression level of Cyclin D1 was significantly increased when fenhexamid and E2 were worked on the BG-1 cells without ICI for 72 hours (see **Figure 3B**). The expression level of Cyclin D1 was increased by 1.2 times after the treatment of fenhexamid and E2 was also increased by 1.5 times. But when fenhexamid and E2 were worked on the BG-1 cells in the combination with ICI, the expression level of Cyclin D1 was similar to it in the control group (see **Figure 3B**). All the results showed that E2 and fenhexamid induced the expression level of Cyclin D1 to be increased was regulated by ER dependent pathway.

The influence of fenhexamid on Cyclin E

The results of Western blot experiment showed that after 24 hours and 72 hours when E2 and fenhexamid (10^{-5} M) were worked on BG-1 cells, the expression level of Cyclin E was significantly increased after 72 hours when it was compared with Cyclin E of the control group. There were significant differences ($P < 0.05$). And it exhibited time-dependent manner. The expressions level of Cyclin E was more significantly enhances with the increase of the incubation time after drug treatment (see **Figure 4A**). The expression level of Cyclin E was no obvious change when it was compared with Cyclin E of control group after the treatment of E2 and fenhexamid in the combination with ICI (10^{-8}) for 24 hours and 72 hours (see **Figure 4A**). And the expression of Cyclin E was extremely increased after the treatment of E2 and fenhexamid without ICI for 72 hours (see **Figure 4B**). The expression level of Cyclin E was increased from 1.2 times to 1.5 times and E2 was increased by 1.5 times after the treatment of fenhexamid on BG-1 cells. But when E2 and fenhexamid (10^{-5} M) were worked on BG-1 cells in the combination with ICI, the expression level of Cyclin E was similar to Cyclin E of the control group (see **Figure 4B**). All the results showed that E2 and fenhexamid induced the expression level of Cyclin E to be increased was regulated by ER dependent pathway.

Discussion

Endogenous hormones such as estrogen, especially E2, had a significant influence on the proliferation of cancer cells which had positive reaction to ER such as BG-1 cells. It could induce transcriptional factors by interacting with estrogen receptor response element directly or indirectly to help the expressions of target genes. Though the affinity of some exogenous substrates in the combination with ER was weaker than entogenous estrogen in the combination with ER, the natural or synthetic exogenous substrates could be changed the endocrine function by combined with ER. So these exogenous substrates could cause estrogen-dependent diseases such as reproductive cancers [16, 17].

Pesticide which could eliminate the insects was used in the agricultural production to improve the quality and quantity of agricultural products. But it could also lead more and more harmful substances in the environment, even lead them into human bodies through food chain. The residual pesticide of vegetables and fruits are the main way into human bodies [3, 12].

The results of cell proliferation experiment according to this study showed that E2 and fenhexamid can significantly help the proliferation of BG-1 cells. The cell proliferation activity of E2 and fenhexamid was not significantly different from that in control group when ER in the com-

bination with receptor antagonist ICI worked on BG-1 cells. In Western blot experiment, the expression levels of Cyclin D1 and Cyclin E which were the essential protein to regulate the cell cycle were all significantly increased when E2 or fenhexamid worked on BG-1 cells without ICI. But the effect of E2 and fenhexamid on Cyclin D1 and Cyclin E disappeared and the expression levels of Cyclin D1 and Cyclin E were similar to those in the control group when ER in the combination with receptor antagonist ICI worked on BG-1 cells. There was no significant difference. In a conclusion, the study results suggested that fenhexamid has obvious estrogen-like effects. It can help the proliferation of BG-1 cells by ER dependency signals just like E2 to regulate the increase of expression levels of Cyclin D1 and Cyclin E, and then to strengthen the proliferation of BG-1 cells. And another signification of this study is that a green, safe living environment is extremely important on the prevention and control of diseases for patients or healthy people.

Disclosure of conflict of interest

None.

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