

Full Length Research Paper

The effect of genistein for preventing granulosa cell injury induced by cisplatin

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Genistein (GEN), the primary isoflavone in legumes, has a well known weak estrogenic effect by binding to estrogen receptors, and widely used for the treatment of ovary disease induced by chemotherapeutics, however, the details of the exact mechanisms was unclear so far, thus, the aim of our study was to find the effect on granulosa cells of ovary induced by cisplatin (CDDP) after using GEN treatment by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method and flow cytometry. The results demonstrated that CDDP could inhibit the proliferation of granulosa cells of ovary by affecting cell cycle S stage. Moreover, CDDP also could arrest cell cycle in G1-M stage, which would evidently increase the number of apoptotic cell. Genistein has the potential to prevent the damaging effect of CDDP and improve the differentiation and proliferations of the cells to make the blockage of the cell cycle disappear, which is related to the dose of GEN and time. The present study provides improvement in understanding the molecular pathogenic mechanism of premature ovarian failure (POF) induced by chemotherapeutics and development of GEN as effective treatment drugs.

Key words: Genistein, Premature ovarian failure, Granulosa cells of ovary, Flow Cytometry.

INTRODUCTION

Premature ovarian failure (POF) is a disorder of multicausal etiology, leading to infertility in women before the age of 40 (McGee and Hsueh, 2000; Rees and Purdie, 2006). With cure rates of cancers in childhood and young women improving, it is likely that the incidence of prematurely menopausal women will rise rapidly (Sklar et al., 2006; Panay et al., 2008; Rebaret et al., 1990). There are more and more patients suffering from ovarian deficiency and infertility caused by chemotherapy, which becomes one of the important etiological factors of POF (Goswami and Conway, 2005; Krishna et al., 2010). The disease model is more complex and difficult to prevent with therapeutics, hence it is necessary to understand molecular mechanisms responsible for premature ovarian

failure caused by chemotherapy and study new technologies and introduce new drugs to prevent and treat the POF. Genistein (GEN) is a phytoestrogen that occurs naturally in the diet and is found in a wide variety of plant-derived foods especially in soybeans and soy-based foods (Park et al., 2010). GEN has a well known weak estrogenic effect by binding to estrogen receptors (Kim et al., 1998). Increasing evidence showed that GEN plays a major role in prevention of cancer (Imhof and Molzer, 2008), osteoporosis (Taku et al., 2010), heart diseases (Sbarouni et al., 2007), and cognitive dysfunction (Thorp et al., 2009). GEN has recently received considerable research attention on the mechanism of their actions in ovarian disease. There is growing interest in the beneficial effects of GEN on postmenopausal symptoms (Suthar et al., 2001). Furthermore, GEN has been intensively investigated in recent years as a chemopreventive agent, mainly against hormonally regulated POF by

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chemotherapy in animal models (Huang et al., 2008). But additional studies are needed to elucidate the pharmacological mechanisms of GEN treatment for POF by chemotherapy.

In light of the existing, yet unresolved views on POF by chemotherapy, in this study, we used granulosa cells of ovary induced by cisplatin (CDDP) to explore the effect of GEN on proliferation of granulosa cells of ovary, which might help us to learn about the effects of GEN on granulosa cells of ovary cell inhibition at cellular level.

MATERIALS AND METHODS

Isolation and culture of granulosa cells of ovary

Immature female Wistar rats were obtained from animal experimental center of Beijing University of Chinese Medicine (China) at 21 days of age, housed in standard cages, and provided food and water *ad libitum*. All protocols were approved by the Institutional Animal Care from Beijing University of Chinese Medicine. Starting on day 22, some animals were injected twice daily with Pregnant mare's serum gonadotropin (PMSG) (Sigma, USA) for up to 48 h to induce follicular development. Animals were euthanized by inhalation of Halothane. Ovaries were aseptically removed to DMEM-F12 tissue culture medium. The ovaries were quickly trimmed of surrounding adipose tissue, bursa, and connective tissue. Primary cultures of rat granulosa cells were established as previously described (Clemens et al., 2000). Rat granulosa cell lines were cultured in DMEM-F12 containing 5% fetal bovine serum (Sigma, USA) until approximately 70% confluent and then used for assays.

MTT assay

The *in vitro* drug sensitivity to cisplatin was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Granulosa cells were plated at a density of 50,000 cells in 96-well plates. They were allowed to recover for 72 h and then exposed to various concentrations of etoposide and carboplatin for 24 to 72 h. Then, drug cytotoxicity was evaluated by using a MTT reduction conversion assay (Sigma, USA). Forty microliters of MTT at 5 mg/ml concentration was added to each well, and incubation was continued for 4 h. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 200 μ l of dimethyl sulfoxide, and absorbance was measured at 490 nm by using a SpectraMAX microplate reader (Molecular Devices, USA). Each combination of cell line and drug concentration was set up in eight replicate wells, and the experiment was repeated three times, then, half maximal inhibitory concentration of cancer cells (IC_{50}) was counted. Cell survival was expressed as absorbance relative to that of untreated controls. Granulosa cells of ovary induced by cisplatin using IC_{50} concentration was determined by MTT assay after treatment of at the concentration of 0.5×10^{-6} , 1×10^{-6} , 2×10^{-6} , 4×10^{-6} mol/L. The detail manuscript was performed according to the above experiment.

Flow cytometry

Confluent of granulosa cells of ovary were seeded in 12-well culture plates at a density of 5×10^5 cells/well. After serum-starvation for 24 h, granulosa cells were incubated with DMEM containing 2×10^{-6} mol/L of genistein and the IC_{50} concentrations of cisplatin for 96 h.

The cells were detached from the plates using 0.1% trypsin, 1% EDTA and fixed with 70% ethanol. After two washes with PBS, granulosa cells were incubated with 1% Triton X-100 and 0.1 mg/mL RNase A at 37°C for 30 min. After centrifugation (1,200 rpm for 5 min at room temperature), the supernatant was discarded and 0.1 mg/mL propidium iodide (PI) was added, and placed on ice for 30 min. After passing through a nylon filter membrane, the cells were analyzed by flow cytometry to determine cell cycle. Each condition was analyzed in quadruplicate and the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used to analyse all group data.

Statistics analysis

The statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way ANOVA test was used to determine the significance of differences among the groups. All values were expressed as mean \pm SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Effects of GEN on cell viability

To determine the effects of cell viability and inhibition induced by cisplatin, the viability of the treated cells was measured by MTT assay. As shown in Table 1, significant cytotoxic effect and cell growth inhibition on cells was showed by cisplatin. Compared with control group, the viability of ovary cells exposed to h cisplatin was significantly inhibited with increasing time. In addition, the viability of ovary cells in high dose cisplatin were significantly increased compared to low dose cisplatin ($P < 0.05$) (Table 1).

IC_{50} of cisplatin was 8.85 μ g/mL by calculation. MTT assay was also performed to determine the effects of genistein on cisplatin induced cell injury and viability. As shown in Table 2, high dose of GEN could significantly decrease cisplatin induced decrease in cell viability compared to low dose of GEN ($P < 0.05$) (Table 2). Low dose of GEN has no significant effect on the cisplatin induced decrease in cell viability.

Cell cycle analysis

Flow cytometry was conducted to analyse cell cycle by GEN. As shown in Table 3, granulosa cells exhibit higher level apoptosis, when they are exposed IC_{50} of cisplatin. Apoptosis was significantly decreased after addition of GEN ($P < 0.05$). Compared with control group, the percentage of cells at G0/G1 phase increased significantly in CDDP + GEN group and CDDP group. In addition, the percentage of CDDP group significantly decreased, compared with the control group at S and G2/M phase, while the percentage of CDDP + GEN group significantly increased compared with the CDDP group and close to control group at S and G2/M phase.

Table 1. Effect on granulosa cells of ovary by cisplatin at different concentration and time.

(µg/ml)	24h		48h		72h	
	Absorbency	Inhibition ratio (%)	Absorbency	Inhibition ratio (%)	Absorbency	Inhibition ratio (%)
0	0.2827±0.0102		0.2914±0.0106		0.3003±0.0109	
0.25	0.2825±0.0111	0.11±0.02	0.2820±0.0122	0.19±0.01	0.2826±0.0121	0.12±0.02
0.5	0.2821±0.0108	0.20±0.03	0.281±0.0098	0.50±0.19	0.2818±0.0098	0.42±0.20
1	0.2765±0.0103*	4.67±1.63*	0.2618±0.0060	7.34±1.50	0.2712±0.0120*	5.76±1.16*
2.5	0.2443±0.0125*	14.34±1.82*	0.2300±0.0098	18.51±1.82	0.2387±0.0105*	16.32±1.54*
5	0.2012±0.0193*	26.56±3.83*	0.1888±0.0116	33.12±5.41	0.1953±0.0116	30.63±4.70
10	0.0753±0.0989*	78.92±6.19*	0.0332±0.1810	88.24±5.62	0.0569±0.1024*	84.30±7.77*

Different mark represent the significant difference at $p < 0.05$.

Table 2. Effect on granulosa cells of ovary by cisplatin combined with genistein at different concentration and time.

Group	Concentration		Inhibition ratio (%)		
	CDDP (µg/mL)	GEN (mol/L)	12 h	24 h	48 h
CDDP	5	0	40.58±4.94	30.24±2.12	36.32±4.11
CDDP+GEN 1	5	5×10^{-6}	36.66±2.75	27.09±3.06	30.71±5.20
CDDP+GEN 2	5	1×10^{-6}	18.78±4.65*	15.80±2.51*	19.00±1.18*
CDDP+GEN 3	5	2×10^{-6}	15.25±2.86*	9.37±3.72*	9.69±3.22*
CDDP+GEN 4	5	4×10^{-6}	6.38±1.04*	5.42±1.71*	5.41±2.04*

Different mark represent the significant difference at $p < 0.05$.

Table 3. Effect on granulosa cells of ovary by cisplatin and cisplatin combined with genistein at different concentration and time,

Group	Cell cycle			
	G ₀ /G ₁	S	G ₂ /M	Apoptosis
Control	61.41±2.35	26.80±1.52	20.13±1.10	2.547±1.05
CDDP	72.36±2.37*	15.47±1.14*	16.57±1.01*	6.46±1.18*
CDDP+GEN	67.31±2.47* ^Δ	26.01±2.02 ^Δ	18.29±1.05	3.33±1.04 ^Δ

Different mark represent the significant difference at $p < 0.05$.

DISCUSSION

Cisplatin (CDDP), an important anti-cancer drug, could cause serious damage to ovary tissue by harming the granulosa cells of ovary. This may cause ovarian deficiency and infertility (Dube et al., 1998). Previous study showed that toxicity was associated with plasma lipid peroxides (Previati et al., 2006). Moreover, other study have shown that CDDP combined with DNA caused cell damage (Olas et al., 2006; Salsbury et al., 2006), which could cause apoptosis to some extent. Our study showed that high dose of CDDP caused apoptosis of granulosa cells of ovary at S phase, which was consistent with previous reports that chemotherapeutics have

significant role in interfering with cell cycle (Horowitz et al., 2004; Crescenzi et al., 2006; Xu et al., 2007; Hara et al., 2006).

Genistein has been shown to have many biological activities, such as anti-cancer, anti-oxidant, anti-inflammatory actions and inhibition of tyrosine-specific protein kinases (Akiyama et al., 1987; Rusin et al., 2010; Park et al., 2010; Zhang et al., 2008). GEN has become a popular candidate for drug development because of these features. In the present study, it was found that high dose of GEN could inhibit damage of granulosa cells of ovary caused by CDDP, which was consistent with previous study that GEN inhibit cell damage by chemotherapeutics (Nynca et al., 2006), however, the detailed

molecular mechanisms of GEN inhibition cell damage was unclear so far, which would depend on further study.

Conclusion

The present study demonstrated that GEN could decrease cell damage of ovary induced by CDDP, which demonstrated that GEN is an effective drug to treat ovarian disorders induced by chemotherapeutics. The current study provides an effective approach for studying the mechanism underlying the pathogenesis of premature ovarian failure at cell level and treating premature ovarian failures. What is more, considering its low toxicity, GEN can be one of the potential drugs for more treatments of human diseases than now.

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