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Full Length Research Paper

Genistein inhibits expression of matrix metalloproteinases-2 (MMP-2) in premature ovarian failure (POF) rats induced with cisplatin intraperitoneal injection

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Matrix metalloproteinases (MMPs) play a pivotal role in ovary disease by degrading multiple elements of the extracellular matrix (ECM). Therefore, the inhibition of MMPs has been suggested to be a promising therapeutic strategy for premature ovarian failure (POF). Genistein, the primary isoflavone in legumes, has a well known weak estrogenic effect by binding to estrogen receptors, and widely used in the treatment of POF; however, MMPs changes after using genistein (Gen) was unclear. The aim of our study was to evaluate changes from MMP-2 of POF rats induced by cisplatin intraperitoneal injection after using different dose of GEN treatment by western-blot and immunohistochemistry technology. The results demonstrated that MMP was significantly down-regulated in middle dose GEN group and high dose of GEN group in comparison to the cisplatin group on protein level ($P < 0.05$), and middle dose GEN group, high dose of GEN group and control group had no significant difference on mRNA level ($P < 0.05$). The present study provides improvement in understanding the molecular pathogenic mechanism of POF and development of Gen as effective treatment drugs.

Key words: Genistein, premature ovarian failure, MMP-2, cisplatin, western-blot, immunohistochemistry.

INTRODUCTION

Premature ovarian failure (POF) generally describes as a syndrome consisting of amenorrhoea, sex steroid deficiency and elevated/menopausal levels of gonadotropins in a woman occurring before the age of 40 (Rees et al., 2006). It could be primary (spontaneous POF) or secondary (induced by radiation, chemotherapy or surgery) (Coulam et al., 1986). With cure rates of cancers in childhood and young women continue to improve, it is likely that the incidence of prematurely menopausal women will rise rapidly (Sklaret al., 2006; Panay et al., 2008; Rebaret al., 1990). The disease model is more complex and difficult to prevent with therapeutics, thereby making it an extreme detriment to the female healthy. Thus, the research of new technologies to prevent and treat the POF has become an urgent task to many researchers.

Hormone replacement therapy (HRT) method was widely performed to improve survival quality in patients with POF, but this method might increase the risk of cancer by estrogen depended (Dietel et al., 2005; Horn et al., 2005), thus, the research of safe and effective hormone replacement drug is important to control and cure POF. Genistein, the primary isoflavone in legumes, has a well known weak estrogenic effect by binding to estrogen receptors (Kim et al., 1998) and is widely used as a protein tyrosine kinase (PTK) inhibitor at pharmacological doses (Akiyamaet al., 1987). Apart from their estrogenic properties, other features could also be involved in their observed diverse action mechanisms, including binding to other nuclear receptors (ERR, PPAR,

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AhR, etc.), antioxidant effects due to their polyphenolic nature, modulation of steroid metabolism or of detoxification enzymes, and anti-cancer (Atteritano et al., 2007; Hertrampf et al., 2007; Yang et al., 2006), therefore, genistein could acted as HRT drug to treat POF by chemotherapy.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play a pivotal role in ovary disease by degrading multiple elements of the extracellular matrix (ECM), including laminin, collagen, and fibrous proteins (Kim et al., 2008; Clark et al., 2008), therefore, the inhibition of MMPs expression might be a promising therapeutic strategy for POF, thus, in the present study, we used rats with POF induced by cisplatin intraperitoneal injection as study objective to evaluate the MMP-2 change after using different dose of GEN treatment by weston-blot and immunohistochemistry technology, which might be a possible beneficial POF induced by chemotherapy treatment.

MATERIALS AND METHODS

Experiment rat and sample preparation

100 IRC rats (3 weeks age, 20-30 g) were studied after 1 weeks of observation. IRC rats were randomly divided into control group (n=20) and experiment group (n=80) after one weeks. The animal model of POF was established by intraperitoneal injection of cisplatin (1g/L, 3.0 mg /kg,qilu pharmaceutical, China) for one week successively in experiment group. Experiment group were randomly divided into low dose of group by gastric perfusion GEN (50 mg/kg, purity >99%, Sigma, USA), middle dose of group by gastric perfusion GEN (100 mg/kg) group, high dose of group by gastric perfusion GEN (200 mg/kg) and POF group (n=20 in each group). Ovary tissues were collected from rats after fed two weeks.

Western blot

In order to detect protein expression level of MMP-2 all subjects, western blot was performed as described in the online supplement**.** The ovary tissues were obtained from each group. Protein homogenates of ovary samples were prepared by RIPA (Bio-ride, USA) on ice for 30 min. Tissue homogenate were centrifuged at 12,000 g for 1 h at 4°C and the protein concentration in the supernatant was determined using BCA protein concentration determination kit according to the manufacturer's protocol and as described in the online supplement. Equal amounts of protein (20 μg) from each sample were loaded and separated into a 7.5% gradient SDS-PAGE under denaturing conditions. Electroblotting proteins were transferred onto nitrocellulose membranes (Amersco, USA). After blocking with 5% nonfat dry milk overnight at 4°C, membranes were incubated for 2 h at room temperature in agitation with the following antibodies: rabbit polyclonal anti-MMP-2 (dilution 1:800; Santa Cruz Biotechnology, Inc), and rabbit polyclonal anti-β-actin (dilution 1:800; Santa Cruz Biotechnology, Inc). Secondary horseradish peroxidase conjugated rabbit anti-goat/ goat anti-rabbit antibodies (Santa Cruz Biotechnology, Inc) were used at 1:3000 dilution for 2 h at room temperature in agitation. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Inc) and scanned using Chemi Imager 5500 V2.03 software. The integrated densities value (IDV) was analyzed with computerized image analysis system (Fluor Chen 2.0) and normalized with that of β-actin.

Immunohistochemical analysis

To detect expression and localization of MMP-2 in ovary tissue of all subjects, immunohistochemical was performed. Frozen ovary tissue samples obtained from rats with POF group, GEN group and control group were processed and cut at 4 μm for slide preparation. The sections were deparaffinized in xylen and rehydrated with graded alcohols. For heat-induced epitope retrieval, the sections were immersed in 0.01 M citrate buffer solution (pH 6.0) for 10 min. Then, they were cooled for 1 h at room temperature and washed in water and phosphate-buffered saline (PBS). Next, 0.3% hydrogen peroxide was applied to block endogenous peroxidase activity, and the sections were incubated with normal goat serum to reduce nonspecific binding. They were then incubated overnight at 4°C with primary rabbit polyclonal anti-rats antibody (1:150; Santa Cruz). Biotinylated goat anti-rabbit serum IgG was used as a secondary antibody. After the sections were washed 3 times in PBS, the sections were stained using the ABC Kit (Santa Cruz Biotechnology, Inc), and the color was developed with diaminobenzidine (DAB). Negative controls were conducted by exchange of primary antibody for PBS. The degree of immunoreactivity was assessed similarly to the system described by Campo et al. (1992). All slides were scored independently by two observers (M.B.R. and O.Y.). Five cases with discordant results were reevaluated to obtain agreement.

Statistical analysis

To calculate the statistical differences all subjects, the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way ANOVA followed by Bonferroni's post hoc test were utilized to determine the significant difference among multiple groups. Student's *t* test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Western blot analysis of MMP-2 expression

In order to detect the protein expression of MMP-2 in rat with POF by GEN treatment, Western blot was conducted. As shown in Figure 1, MMP-2 protein levels in high dose GEN and middle dose of GEN group were significantly decreased after GEN treatment compared to POF group ($P<0.05$), moreover, POF group were significantly higher than control group ($P<0.05$), and high dose GEN group, middle dose of GEN group and control group had no significantly difference. These results showed that high dose GEN group, middle dose of GEN treatment could down-regulate MMP-9 on protein expression.

Immunohistochemistry analysis of MMP9 expression

The immunostainings were performed to detect MMP-2 protein expression of using a multiheaded microscope.

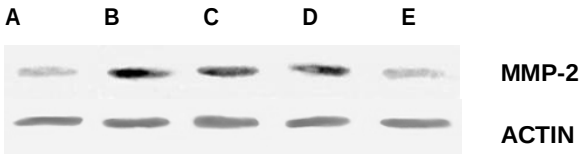

Figure 1. The expression of MMP-2 in rats with POF after genistein treatment in protein level at different group. (A) control group; (B) POF group; (C) low dose of GEN group; (D) middle dose of GEN group; (E) high dose of GEN group.

Table 1. The integrated density value of MMP-2 protein in rats with POF after genistein treatment in protein level at different group, different mark represent the significant difference at p<0.05.

Group	The integrated density value of MMP-2
	MMP-2
Control	0.511±0.023
Low dose	0.691±0.024*
Middle dose	0.564±0.028△
High dose	0.550±0.031△
POFmodel	0.722±0.031*

Table 2. The expression of MMP-2 in rats with POF after genistein treatment in protein level at different group by immunohistochemical staining, different mark represent the significant difference at p<0.05.

Group	OD
	MMP-2
Control	187.85±15.92
Low dose	251.06±15.41△
Middle dose	236.35±14.86△
High dose	215.54±13.96△
POFmodel	289.76±16.12*

All MMP-2 immunostaining results was illustrated in Table 1. The result showed that there were significantly differences (P<0.05) MMP-2 expression between control, high dose of GEN group, and middle dose of GEN group (P < 0.05) and MMP-2 protein expression levels in GEN group were significantly down-regulate in compared to POF group (P<0.05). These results indicated that MMP-2 expression was decreased in GEN treatment group, which was compatible with result of MMP-2 protein expression decreasion using Gen treatment.

DISCUSSION

MMP family are involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as all kinds of cancer (Atteritano et al., 2007; Hertrampf et al., 2007; Yang et al., 2006) and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. In the present study, we found that rats with POF induced by cisplatin group were significantly higher than control group (P<0.05), which showed that MMP protein of ovary was activated by cisplatin, therefore, ECM of ovary was breakdown, which might be an important reasons for POF occurrence.

MMPs are key proteases modulating the proliferation and invasion of ovarian granulosa cells (Bello et al., 2004). Thus, intervention of MMP expression has been suggested as a promising therapeutic strategy for POF. In the present study we demonstrated that an isoflavone metabolite, genistein, specifically inhibited the expression of MMP-2 in ovary tissue of rats with POF induced by cisplatin, which might be a key reason that POF was treated by genistein (Table 2). Moreover, 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). Genistein has become a popular candidate for drug development because of these features. These features of genistein also help to treat POF, however, the underlying molecular mechanisms of POF treated by genistein, which would depend on further study.

In conclusion, the present study demonstrated that MMP-2 was significantly down-regulated in rats with POF after genistein treatment in comparison to the POF rats induced by cisplatin, which demonstrated that genistein could decrease MMP-2 expression by some effective drug component. The current study provides an effective approach for studying the mechanism underlying the pathogenesis of premature ovarian failure and treating premature ovarian failure method.

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