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THE EXTRACTS OF PACIFIC OYSTER (*CRASSOSTREA GIGAS*) ALLEVIATE OVARIAN FUNCTIONAL DISORDERS OF FEMALE RATS WITH EXPOSURE TO BISPHENOL A THROUGH DECREASING FSHR EXPRESSION IN OVARIAN TISSUES

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## Abstract

**Background:** Bisphenol-A (BPA) is one of the widespread industrial compounds, which has adverse effects on animal and human health. The study was aimed to explore the effects of *Crassostrea gigas* extracts (CGE) in alleviating ovarian functional disorders of female rats with exposure to BPA and the underlying possible mechanism.

**Materials and methods:** Eighteen four-week-old female Sprague-Dawley (SD) rats were randomly divided into BPA group (50mg/kg BPA), BPA+CGE group (50mg/kg BPA+50mg/kg CGE), and control group (equivalent dosage of vehicle) with 6 rats in each group. After a 6-week treatment ended, the serum levels of estradiol (E<sub>2</sub>), follicle stimulating hormone (FSH), luteinizing hormone (LH) were measured by using commercial standard assay kits. The expression levels of FSH receptor (FSHR) in the rat ovarian tissues were respectively detected by immunohistochemistry and Real-time PCR.

**Results:** CGE treatment markedly increased E<sub>2</sub> levels and decreased FSH levels in the serum (P<0.05), however, the alterations of serum LH levels were not significant (P>0.05). The protein and mRNA expression levels of FSHR were the lowest in the ovaries of control rats and the highest in BPA rats (P<0.05). CGE treatment markedly decreased the expression levels of FSHR in the ovarian tissues (P<0.05).

**Conclusions:** *Crassostrea gigas* successfully alleviates ovarian functional disorders of female rats with exposure to BPA partly through decreasing FSHR expression levels in the ovarian tissues.

**Keywords:** *Crassostrea gigas*, bisphenol A (BPA), follicle stimulating hormone receptor (FSHR).

## Introduction

Bisphenol-A (BPA) is one of the widespread industrial compounds, which is commonly used in the production of food and drink packaging, plastic consumer products, and dental materials (Biedermann et al., 2010; Ozaki et al., 2004), consequently, the exposure of human beings to BPA is widespread and continuous (Borrell, 2010; Diamanti-Kandarakis et al., 2010; Vandenberg et al., 2007). BPA metabolites were found in the urine of approximately 93% of the U.S. general population according to a National Health and Nutrition Examination Survey (Calafat et al., 2008). As BPA can bind to several hormone receptors, including estrogen and thyroid receptors, it is classified as an endocrine disruptor (Moriyama et al., 2002; Zoeller et al., 2005). BPA has adverse effects on animal and human health (Vandenberg et al., 2012; Welshons et al., 2006). It has been found that BPA has negative effects on ovarian steroidogenesis, folliculogenesis, as well as ovarian morphology and the female gonad is a particularly sensitive target of BPA disruption (Markey et al., 2003; Newbold et al., 2007; Schonfelder et al., 2002). However, little therapeutic intervention has found to alleviate the ovarian functional disorders induced by exposure to BPA.

The Pacific oyster (*Crassostrea gigas*) is one of the most economically important bivalves, which is distributed worldwide (Yamaura et al., 2008). Pacific oyster (*Crassostrea gigas*) has been used as traditional complementary and alternative medicine with long history {Yang, 2012

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#773} {Han, 2007 #779}. The scope of the commercial fishery for *Crassostrea gigas* has markedly increased since 1993, when *Crassostrea gigas* was introduced to the west coast of the United States from Japan. Rapid progresses have been made on the improvement of economic traits for *Crassostrea gigas*, including development, growth and pathogen-resistance (Huhtaniemi and Themmen, 2005; Peluso and Steger, 1978). In 2007, the production has reached 4.2 million tons worldwide (FAO, 2009). As *Crassostrea gigas* extracts (CGE) can significantly increase the levels of glutathione and activity of glutathione S-transferase (GST) (Gate et al., 1999; Gate et al., 1998), a key factor of improving steroid hormone metabolism in ovaries (Sesh et al., 2001), the present study was designed to explore the effects of CGE in alleviating ovarian functional disorders of female rats with exposure to BPA and the possible underlying mechanism.

## Materials and methods

### Materials

The specimens of *Crassostrea gigas* were collected from the waters around Zhoushan, Zhejiang Province, China. The specimens were stored at  $-20^{\circ}\text{C}$  after the fresh whole bodies were taken away from the shell. The shelled *Crassostrea gigas* (2.5kg) were chopped and homogenized, which were then extracted with hot water ( $75^{\circ}\text{C}$ ) for 3.5 hours. After cool to room temperature, they were filtered with Celite powder and filter paper. The filtrate was used as CGE in the present study. BPA were provided by Sigma-Aldrich Co., U.S.A.

### Animals and groups

Eighteen four-week-old female Sprague-Dawley (SD) rats were provided by Experimental Animal Centre, School of Medicine, Zhejiang University (Hangzhou, China). The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and maintained at a constant temperature of  $25^{\circ}\text{C}$  and humidity of 55%. The rats were fed with standard pelleted food and plain tap water *ad libitum* for 1 week. The present study was performed according to the National Research Council's protocol for the care and use of laboratory animals and was approved by the ethics committee. All of the rats presented at least two classic estrous cycles before the individual treatments below. They were randomized with the use of a randomization chart constructed in Microsoft Excel that randomized numbers into three groups. The rats were then randomly divided into BPA group, BPA+CGE group, and control group with 6 rats in each group. The rats in BPA group were orally administrated BPA at 50 mg/kg once daily for 6 consecutive weeks. The rats in BPA+CGE group were orally administrated BPA at 50 mg/kg and CGE at 50mg/kg once daily for 6 consecutive weeks. The rats in control group were orally administrated the equivalent dosage of vehicle once daily for 6 consecutive weeks. All the compounds were first dissolved in 100% ethanol (EtOH, Pharmaco), and then sesame oil at a ratio of 10% EtOH and 90% oil (Patisaul et al., 2006). The vehicle was a mixture of 10% EtOH and sesame oil. The dose of BPA used in the present study is equivalent to the lowest level of observed adverse effects from oral administration (FAO/WHO, 2011).

### Samples collection and measurement

During the above treatment period, none of the rats died. On the next day following the above 6-week treatment, the rats were anaesthetized with urethane (1.2 g/kg, intra-peritoneally) after fasting for 12 h. The blood samples were then drawn from the hepatic portal vein into heparinized injectors. The collected samples were centrifuged at 3000 rpm in  $4^{\circ}\text{C}$  for 12 min. The supernatant serums were then transferred to clean EP tubes and stored at  $-80^{\circ}\text{C}$  until assay. The rats were sacrificed after the blood samples were collected. The ovaries were rapidly removed and washed with physiological saline. The left ovary was sliced and the tissue slices were fixed in 10% buffered-neutral formalin for 24 hr for histological examinations. The other ovary were frozen and stored at  $-80^{\circ}\text{C}$  until the time of the assay. The serum levels of estradiol ( $\text{E}_2$ ), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by using commercial standard assay kits (Wuhan Boster Bio-Engineering Limited Company, Wuhan, China). All the measurements were performed in duplicate and were conducted according to the manufacturer's instruction. Both intra- and inter-assay coefficients of variation were less than 10%. FSHR mRNA expression level in the ovary was measured by Real-time PCR.

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## Immunohistochemistry

The fixed ovary tissue slices were embedded in paraffin, sectioned, de-paraffinized, rehydrated, and then were cut into sections and mounted on slides. The sections were incubated with the polyclonal FSHR antibody (1:100 dilution; Sigma, St Louis, MO, USA) at 4°C overnight. The biotinylated secondary antibody and horseradish peroxidase (HRP) conjugated streptavidin were added onto the ovary section after the slides were washed twice with TBS. The expression was visualized with 3,3-diaminobenzidine (DAB) substrate and observed under Eclipse E400 microscopy (Nikon, Tokyo, Japan), which were then analyzed using Image-Pro Plus 6.0.

## Detection of FSHR mRNA expression in ovarian tissues with Real-time PCR

Total RNA was isolated with the RNAiso<sup>TM</sup> Reagent (TAKARA, Dalian, China) according to the instructions of the manufacturer. The purity and concentration of RNA were detected by NanoDrop<sup>®</sup>ND-100 Spectrophotometer (Thermo Fisher Scientific Inc, USA). The cDNA was prepared from 500ng of total RNA by reverse transcription with the PrimeScript<sup>TM</sup> RT reagent Kit (Perfect Real Time, TAKARA, Dalian, China). The samples of cDNA were then diluted in DNase- and RNase-free water at a proportion of 1:3 before further analysis. Quantitative real-time PCR was performed by using the iCycler iQ Real-Time Detection System (Bio-Rad). The rat FSHR gene specific primers were provided by Sangon, Shanghai, China. The sequences of the primers are as follows. FSHR Forward: TTTACTTGCTGGAAGCGACTAA, Reverse: CCCAGGCTCCTCCACACA; GAPDH Forward: GCAAGTTCAACGGCACAG, Reverse: CGCCAGTAGACTCCACGAC. PCR reactions were performed using 2μL of cDNA, 10 μM of each primer, and 2×SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (TAKARA) in 20-μL reactions. Thermal cycling conditions were 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60.0°C for 30 seconds. Gene starting quantity was based on the cycle threshold (Ct) method. Each value was normalized to GAPDH to control for the amount of the input cDNA. The threshold cycle value for GAPDH mRNA was subtracted from that of FSHR gene, and the mRNA levels of FSHR gene were expressed as  $2^{-\Delta Ct}$ .

## Statistical analysis

The data were analyzed with Statistical Package for Social Sciences (SPSS 15.0 for Windows). Analysis of variance (ANOVA) was employed for analyzing all the data. A 5% significance level ( $P < 0.05$ ) and two-tailed tests were used for all hypothesis tests.

## Results

### Serum levels of E<sub>2</sub>, FSH and LH

As shown in Figure 1 A and B, the serum E<sub>2</sub> levels of control group were significantly higher, and the serum FSH levels were markedly lower than BPA group and BPA+CGE group ( $P < 0.05$ ). The serum LH levels of control group were significantly higher than BPA group ( $P < 0.05$ ), however, there were no significant differences between BPA+CGE and BPA groups ( $P > 0.05$ ). When comparing with BPA group, the serum levels of E<sub>2</sub> in BPA+CGE group were significantly increased and the serum levels of FSH were significantly decreased ( $P < 0.05$ ), indicating that CGE has positive effects in alleviating ovarian functional disorders of female rats with exposure to BPA.

### FSHR protein expression in rat ovaries detected by immunohistochemistry

As shown in Figure 2, the protein expression levels of FSHR were the lowest in the rat ovaries of control group and the highest in the ovaries of BPA rats ( $P < 0.05$ ). CGE treatment significantly decreased the expression levels of FSHR in the ovarian tissues ( $P < 0.05$ ).

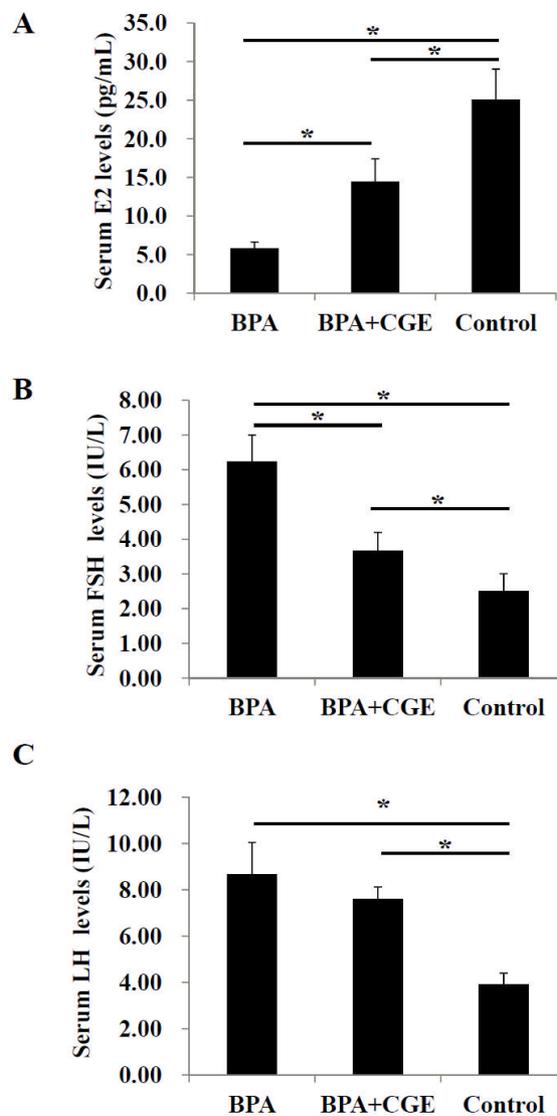
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### FSHR mRNA expression in rat ovaries detected by Real-time PCR

As shown in Figure 3, the expression levels of FSHR mRNA in the rat ovaries from Control group were significantly lower than those of BPA group and BPA+CGE group ( $P<0.05$ ). Moreover, the FSHR mRNA expression levels in the ovaries of rats in BPA+CGE group were significantly lower than BPA group ( $P<0.05$ ).

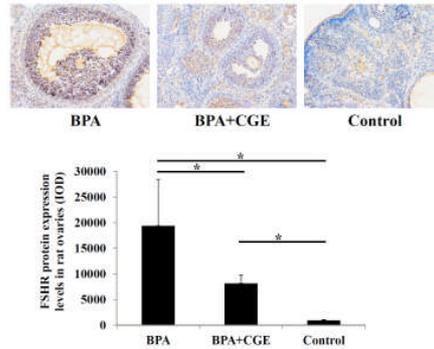
### Discussion

According to a research from Japan, the mean BPA level detected in the follicular fluid was  $2.4\pm 0.8$  ng/ml (Ikezuki et al., 2002). The levels of BPA in polycystic ovary syndrome (PCOS) women were found to be higher than the BMI-matched healthy women and the BPA levels were positively and strongly associated with hormonal and metabolic abnormalities (Diamanti-Kandarakis et al., 2009). The women suffered from ovarian functional disorders induced by BPA exposure would undoubtedly benefit if effective extracts from natural products can be developed.

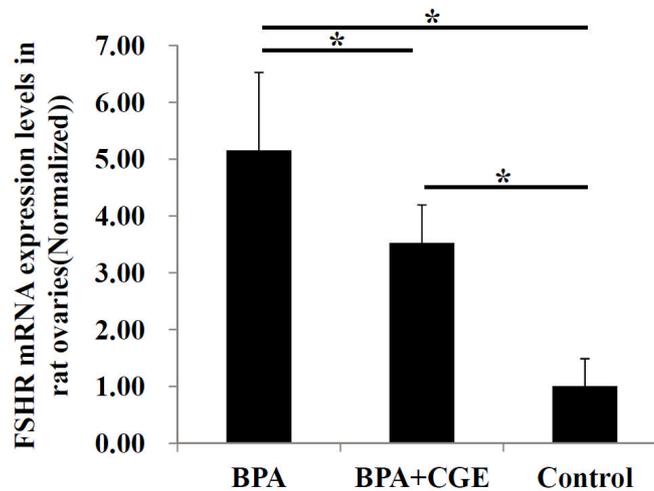


**Figure 1:** Serum levels of sex hormones. (A) Estradiol ( $E_2$ ). (B) Follicle stimulating hormone (FSH). (C) Luteinizing hormone (LH). Bisphenol A (BPA) group (50mg/kg BPA); BPA+*Crassostrea gigas* extracts (CGE) group (50mg/kg BPA+50mg/kg CGE); Control group (equivalent dosage of vehicle). Data were shown as mean  $\pm$  SD. ( $n=6$  in each group). The significant difference was set at \*  $P<0.05$  (ANOVA).

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**Figure 2:** Alterations of expression levels of FSH receptor (FSHR) in rat ovaries detected by immunohistochemistry (A) the representative photomicrographs (200 $\times$ ). (B) FSHR expression levels. IOD: Integrated optical density. BPA group (50mg/kg BPA); BPA+CGE group (50mg/kg BPA+50mg/kg CGE); Control group (equivalent dosage of vehicle). Data were shown as mean  $\pm$  SD. (n=6 in each group). The significant difference was set at \* P<0.05 (ANOVA).



**Figure 3:** FSHR mRNA expression levels in rat ovaries detected by Real-time PCR. BPA group (50mg/kg BPA); BPA+CGE group (50mg/kg BPA+50mg/kg CGE); Control group (equivalent dosage of vehicle). Data were shown as mean  $\pm$  SD. (n=6 in each group). The significant difference was set at \* P<0.05 (ANOVA).

CGE was found to protect human epithelial cells against oxidative stress and increase GST activity in organs of rats (Gate et al., 1999; Gate et al., 1998). The activity of GST increased with the increase in size of the follicles from small to large follicles of follicular phase ovary and from small to medium follicles of luteal phase ovary in goat and sheep, thereafter it decreased in large follicles of luteal phase ovary, indicating GST plays a functional role in the steroid hormone metabolism in the ovaries (Sesh et al., 2001). GST class pi has dual protective role in the rat ovaries, including inhibition of JNK-initiated apoptosis and metabolism of 4-vinylcyclohexene diepoxide, an occupational chemical that selectively destroys ovarian small pre-antral follicles in rats and mice via apoptosis (Keating et al., 2010). A significant decrease in glutathione level and increased DNA damage as well as susceptibility of DNA to oxidative stress were found in PCOS women (Dinger et al., 2005). We found in the present study that CGE treatment markedly increased E<sub>2</sub> levels and decreased FSH levels in the serum.

FSHR/FSHR transcripts were firstly discovered in embryonic gonads around Embryonic Day 20.5 in females (Dankbar et al., 1995; Rannikki et al., 1995). In the ovarian granulosa cells, temporal changes in FSH signaling regulate the main transcriptional, metabolic and hormonal activities, which are important for the proliferation and differentiation events required for follicular growth and oocyte maturation (Dunkel et al., 1994; Simoni et al., 1997). Although confirmatory studies with independent samples are needed, FSHR has been found to be closely associated with the increased genetic susceptibility to PCOS (Du et al., 2010). In the rodent ovary, the expression of FSHR was found to coincide with formation of primary follicles and follicular development through the preantral stage, and the initial full-length transcripts as well as the hormone binding were observed around Postnatal Day 3, which then continued to increase through Postnatal Day 21 (O'Shaughnessy et al., 1994; Sokka

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and Huhtaniemi, 1990). In goose, FSHR was also found to mediate the response of the goose ovary to FSH during the developmental and egg laying stages, and especially during the latter (Kang et al., 2010). FSHR is directly regulated by a combination of transcriptional and posttranscriptional mechanisms induced by FSH and activin in the ovary (Nakamura et al., 1993; Nakatani et al., 1991; Woodruff et al., 1988). We found that CGE treatment markedly decreased the expression levels of FSHR in the ovarian tissues.

It is then concluded that *Crassostrea gigas* successfully alleviates ovarian functional disorders of female rats with exposure to BPA partly through decreasing FSHR expression in the ovarian tissues. More studies should be performed to explore other possible mechanism underlying *Crassostrea gigas* alleviating ovarian functional disorders of female rats with exposure to BPA in the near future.

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