

# Effects of Fenvalerate on Steroidogenesis in Cultured Rat Granulosa Cells<sup>1</sup>

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**Objective** This study was designed to examine the *in vitro* effects of fenvalerate on steroid production and steroidogenic enzymes mRNA expression level in rat granulosa cells. **Methods** Using primary cultured rat granulosa cells (rGCs) as model, fenvalerate of various concentrations (0, 1, 5, 25, 125, 625  $\mu\text{mol/L}$ ) was added to the medium for 24 h. In some cases, optimal concentrations of 22(R)-hydroxycholesterol (25  $\mu\text{mol/L}$ ), Follicle stimulating hormone (FSH, 2 mg/L), or 8-Bromo-cAMP (1 mmol/L) were provided. Concentrations of 17 $\beta$ -estradiol( $E_2$ ) and progesterone ( $P_4$ ) in the medium from the same culture wells were measured by RIA and the steroidogenic enzyme mRNA level was quantified by semi-quantitative RT-PCR. **Results** Fenvalerate decreased both  $P_4$  and  $E_2$  production in a dose-dependent manner while it could significantly stimulate rGCs proliferation. This inhibition was stronger in the presence of FSH. Furthermore, it could not be reversed by 22(R)-hydroxycholesterol or 8-Bromo-cAMP. RT-PCR revealed that fenvalerate had no significant effect on 3 $\beta$ -HSD, but could increase the P450 $_{\text{scc}}$  mRNA level. In addition, 17 $\beta$ -HSD mRNA level was dramatically reduced with the increase of fenvalerate dose after 24 h treatment. **Conclusion** Fenvalerate inhibits both  $P_4$  and  $E_2$  production in rGCs. These results support the view that fenvalerate is considered as a kind of endocrine-disrupting chemicals. The mechanism of its disruption may involve the effects on steroidogenesis signaling cascades and/or steroidogenic enzyme's activity.

**Key words:** Endocrine disruptor; Pyrethroid; Steroidogenesis; Granulosa cell; Rat

## INTRODUCTION

The last 50 years have witnessed growing scientific concerns and public debate over the potential adverse effects that may expose wildlife and humans to many types of man-made chemicals that have the potential to alter the normal functioning of their endocrine systems<sup>[1-2]</sup>. They are known as endocrine-disrupting chemicals (EDCs) or environmental endocrine disruptors (EEDs). Among the classes of EDC, pyrethroid pesticides are drawing increasing attention since they are commonly used for the control of agricultural and indoor pests<sup>[3]</sup>. They are rapidly degraded in the environment, so they have largely replaced organochlorine pesticides which can bioaccumulate and biomagnify. In China, pyrethroid and organophosphorus pesticides are the most popular pesticides used in agriculture. Unfortunately, a few studies have shown that

pyrethroid pesticides can cause dysfunction in reproductive system. Several pyrethroid compounds, such as bioallethrin, fenvalerate, fenothrin, fluvalinate, permethrin and resmethrin, appear to competitively inhibit testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations<sup>[4]</sup>. Go *et al.*<sup>[5]</sup> and Chen *et al.*<sup>[6]</sup> reported that pyrethroid pesticides(d-trans-allethrin, fenvalerate, sumithrin, permethrin and cypermethrin) exerted estrogenic activities in MCF-7 human breast carcinoma cell lines. Although limited, these reports suggest that some pyrethroid compounds are capable of disrupting endocrine function.

Fenvalerate is not only a commonly used agricultural pesticide, but also is listed as one of the EDCs in the environment, workplace and home that pose the most significant potential threat to human health (State Environmental Protection Administration of China and US Endocrine Disruptor Scree-

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ning and Testing Advisory Committee (EDSTAC) of EPA). Humans and animals may be directly exposed to high concentrations of fenvalerate during its manufacture or use as well as to mixtures of pesticides at low concentrations that may interact to produce additive or synergistic effects. Furthermore, fenvalerate is a highly lipophilic compound, thus concentrating to a higher degree in adipose tissue, testes, and ovarian follicular fluid. The pesticides in follicular fluid may impair fertility by altering follicular growth and hormone biosynthesis or by changing granulosa cell-oocyte interactions<sup>[7]</sup>.

The steroid hormones, 17 $\beta$ -estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>), are critical for normal uterine function, establishment and maintenance of pregnancy, and mammary gland development<sup>[8-9]</sup>. Although pesticides may change serum steroid hormone levels by increasing or decreasing steroid catabolism and elimination, several studies have demonstrated that these compounds can directly affect steroid hormone production. In the ovarian, steroid production is controlled by the action of gonadotropins on steroidogenic cell surface receptors and the activation of the cAMP-dependent protein kinase (PKA), and other protein kinases<sup>[10,11]</sup>. Steroidogenesis involves several enzymes of the cytochrome P450 (P450s) family and hydroxysteroid dehydrogenases (HSDs) that catalyze key reactions utilizing cholesterol for the synthesis of progesterone, androgens, and estrogens<sup>[12-13]</sup>. Thus, it is possible that the adverse effect of pesticides on steroid hormone levels might be explained by either a direct action of pesticides on the posttranslational modification of the steroidogenic enzymes or through changes in gene expression. Therefore, the present study was performed to determine the effects of fenvalerate on steroid hormone production and steroidogenic enzyme mRNA levels in primary cultures of rat granulosa cells (rGCs).

## MATERIALS AND METHODS

### *Chemicals*

Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham's (DMEM/F-12 1:1) was purchased from HyClone Life Sciences Company (Logan, Utah). Medium 199, antibiotic (10 000 U/mL penicillin G sodium, 10 000 U/mL streptomycin) and *Trizol* reagent were obtained from GIBCO BRL (Grand Island, NY). Plastic culture plates were purchased from Falcon (Lincoln Park, NJ) and Costar (Corning, NY). 8-Bromo-cAMP was purchased from CalBiochem (La Jolla, CA). Pregnant mare serum gonadotropin (PMSG), androstenedione, Bovine

serum albumin (BSA), 22(R)-hydroxycholesterol (22R-HC), FSH, DMSO and fenvalerate (99.9%) were obtained from Sigma Chemical Co. (St. Louis, MO). The others were purchased from Shanghai Biochemical Reagent Co. Ltd.

### *rGC Culture*

rGCs were collected as described previously with a minor modification<sup>[14]</sup>. Briefly, immature (18-22 days old) female Sprague-Dawley rats were injected sc with administered 40IU PMSG. Twenty-four h later, animals were sacrificed by cervical dislocation. Ovaries were removed and placed in ice-cold Medium 199 supplemented with 0.1% BSA and 10 mmol/L HEPES (containing 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, and 2 mmol/L L-glutamine). Ovaries were cleaned of bursa and other extraneous tissues, and GCs were collected from the surrounding media following follicle puncture. Cells were centrifuged (500 $\times$ g) and resuspended and plated in DMEM:F12 containing 0.2% BSA. Cell number and viability were determined by trypan blue exclusion using a hemacytometer. Aliquots of rGC (2 $\times$ 10<sup>5</sup> viable cells/well) were placed in 24-well culture plates. All GCs were incubated in a final volume of 1 mL DEME:F12/well containing 0.5  $\mu$ mol/L androstenedione, and GCs identified as controls did not receive treatments in addition to androstenedione and chemical solvents. Cultures were conducted at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air.

### *Treatment of Cells*

After rGCs were cultured for 6 h, various concentrations of fenvalerate (0, 1, 5, 25, 125, 625  $\mu$ mol/L) were added to the medium. In some studies, optimal concentrations of 22R-HC (25  $\mu$ mol/L), FSH (2 mg/L), or 8-Bromo-cAMP (1 mmol/L) were provided. Then cells were incubated for 24 h. All treatments were performed in serum-free media. Final concentrations of DMSO and ethanol used as chemical solvents were less than 0.4% and included in controls. At the end of all experiments, medium samples were taken and stored at -20°C for the tests of E<sub>2</sub> and P<sub>4</sub>. Total protein of each sample was determined by a modification of the Bradford method.

### *Assessment of Cellular Viability*

Cellular viability was assessed by assessment of cellular dehydrogenases using the ability of cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasolium bromide (MTT) into formazan during 4 h incubation<sup>[15]</sup>. As described previously, rGCs were

plated into 96-well plates at an initial concentration of  $5 \times 10^4$  viable cells/well. Cells were allowed to attach for 6 h; then the medium was removed and replaced by the medium containing a range of concentration of fenvalerate. After 24 h the medium was removed from the wells, and MTT (5 mg/mL PBS buffer) solution was added for 4 h of incubation. Then the solution was removed and 200  $\mu$ L DMSO per well was added. After 10 min of shaking, the absorbance was determined at 490 nm by CERES908 (Bio-Tek Instruments, Inc., Winooski, VT).

#### RIA of $E_2$ and $P_4$

Concentrations of  $E_2$  and  $P_4$  in the medium from the same culture wells were measured using  $^{125}\text{I}$ - $P_4$  and  $^{125}\text{I}$ - $E_2$  Coat-A-Count RIA kits (Beijing North Institute of Biological Technology). Quantitation of progesterone in the medium was performed after concentrated by lyophilization. All drugs used in these experiments were tested for their possible cross reactivity with the antisera used, but none was detected. According to the specification sheets provided, the anti- $P_4$  antibody cross-reacted 1.8% with  $20\alpha$ -dihydroprogesterone, 2.2% with 11-deoxycorticosterone, and 1.3% with  $5\beta$ -pregnan-3, 20-dione. The cross-reactivity of pregnenolone,  $17\alpha$ -hydroxyprogesterone, and testosterone was less than 0.4% respectively. The assay detection limit was 0.2 ng/mL. Inter- and intraassay coefficients of variation were <10% and <15%, respectively. The anti- $E_2$  antibody cross-reacted 0.1% with estrone, 0.01% with testosterone, and less than 0.1% with other estrogens and androgens respectively. The assay detection limit was 5 pg/mL. Inter- and intraassay coefficients of variation were <10% and <15%, respectively.

#### RT-PCR Analysis

Cells grown in 6-well culture plates were treated as described for 24 h. Total RNA was prepared by *Trizol* extraction (1mL/well). Further procedures were conducted according to the manufacturer's instructions. RNA samples were precipitated in 70% ethanol and stored until use. Dried RNA pellets were dissolved in water containing 0.1% diethylpyrocarbonate (DEPC), and quantified by measuring the absorbency at 260 nm. Aliquots containing 2  $\mu$ g RNA were assayed by relative-quantitative RT-PCR procedure as previously described<sup>[16-17]</sup>. Briefly, RT was performed for 60 min at 42°C using 1  $\mu$ g oligod (T)<sub>18</sub> primers (Pharmacia No. 27-7858, Piscatway, NJ), 0.5 mmol/L dNTPs and 10 unit of AMV reverse transcriptase (Promega No. M510, Madison, WI). PCR was conducted in the presence of 2  $\mu$ L RT

product, all four dNTPs (100  $\mu$ mol/L) and 5 pmol of the appropriate oligonucleotide primers (Table 1). Oligonucleotide primers for the ribosomal protein S16 (RPS16) served as an internal control. Whole PCR reaction mixture volume was 50  $\mu$ L. After denaturation at 94°C for 2 min, the mixture was subjected to 26 thermal cycles in a programmed temperature control system (PTC-200, MJ Research, Inc., MA) as Follows: (1) P450scc,  $3\beta$ -HSD and RPS16: at 94°C, for 30 sec; at 65°C, for 45 sec; at 72°C for 1 min; (2)  $17\beta$ -HSD: at 94°C, for 30 sec; at 57.4°C, for 45 sec; at 72°C for 1 min. Both thermocycler protocols were terminated with a 7-min extension phase at 72°C. PCR products were analyzed on 2% agarose gel via electrophoresis and quantitated by densitometry using a computing densitometer (FR-200, Shanghai FURI Science & Technology Co., LTD). All mRNA levels were normalized to RPS16 mRNA, and results were expressed as the ratio of target gene/RPS16 in arbitrary units.

TABLE 1  
PCR Primer Sequences.

mRNA	Oligonucleotide Sequences (5'-3'; sense/antisense)	Length
P450scc	AGAAGCTGGGCAACATGGAGTCAG TCTCATCCCAGGCAGCTGCATGGT	546bp
$3\beta$ -HSD	ACTGGCAAATTCTCCATAGCC TTCTCCCAGCTGACAAGTGG	402bp
RPS16	TCCAAGGGTCCGCTGCAGTC CGTTCACCTTGATGAGCCCATT	100bp
$17\beta$ -HSD	GAGTGGATGTACTGGTCTGT GGTTTATCTGATGCAGACCATC	747bp

#### Analysis of the Data

All values were expressed as  $\bar{x} \pm s$  when appropriate. The primary statistical tool used in data analysis was a one-way analysis of variance (ANOVA) model with fenvalerate as a treatment factor. In all cases, the level of statistical significance was set at  $P < 0.05$ . All statistical procedures were performed with SAS statistical software packages (SAS institute, Cary, NC).

## RESULTS

#### Assessment of Cell Viability in Cultured rGC

Fig. 1 shows the results obtained from the cell viability assessment that was made at 12 h and 24 h during the culture period. Interestingly, with the dosage of fenvalerate increased, there was no inhibition on cell viability. Conversely, the absor-

bance was ascending with the increase of dosage. At 12 h fenvalerate significantly stimulated rGCs proliferation only at the doses of 125 and 625  $\mu\text{mol/L}$ . But at 24 h, this stimulatory effect was observed at each dose.

#### Effects of Fenvalerate on Basal Steroid Hormones Production in rGCs

For dose-response study, rGCs were cultured in 24-well plates with or without fenvalerate. Since fenvalerate could induce rGCs proliferation significantly at 24 h (Fig. 1), the concentration of steroid hormone at each dose was normalized to total protein level. Results showed that this pesticide decreased both  $\text{P}_4$  and  $\text{E}_2$  production in a dose-dependent manner (Fig. 2). At 125  $\mu\text{mol/L}$  it significantly ( $P < 0.01$ ) reduced  $\text{P}_4$  and  $\text{E}_2$  levels by 68.6% and 32.4%, respectively. We chose the mid-doses (25  $\mu\text{mol/L}$  and 125  $\mu\text{mol/L}$ ) for the remaining studies.

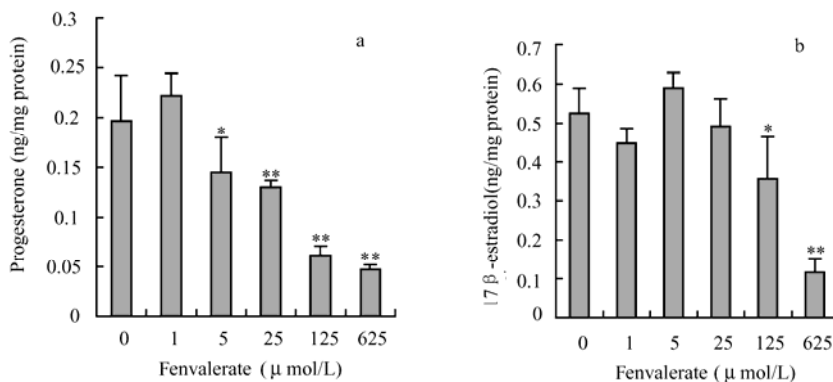


FIG. 2. Effects of fenvalerate on steroidogenesis in cultured rGCs. Cells were treated with fenvalerate at various concentrations of for 24 h in 24-well plates. The steroid hormone levels were assayed as described in Materials and Methods. Each data point is the  $\bar{x} \pm s$  of three replicates in a single experiment which was performed three times. Statistically significant differences are designated with (\*)  $P < 0.05$  or (\*\*)  $P < 0.01$ .

#### Effects of Fenvalerate on FSH-, 8-Bromo-cAMP- and 22R-HC-stimulated Steroid Hormones Production in rGCs

The effects of fenvalerate on FSH-stimulated steroid hormone secretion are shown in Fig. 3. The inhibition of fenvalerate to FSH-stimulated steroidogenesis was more significant than to basal steroidogenesis. As shown in Fig. 3a, at 25  $\mu\text{mol/L}$  and 125  $\mu\text{mol/L}$  fenvalerate reduced basal  $\text{P}_4$  production by 34.2% and 68.6%, respectively, but it reduced FSH-stimulated  $\text{P}_4$  production by 90.5% and 95.3%, respectively. Similarly in Fig. 3b, fenvalerate reduced FSH-stimulated  $\text{E}_2$  production by 45.1% and 59.2%, respectively.

To determine if the inhibitory effects of fenvalerate on FSH-stimulated steroidogenesis in rGCs might be due to an inhibition of cAMP production,

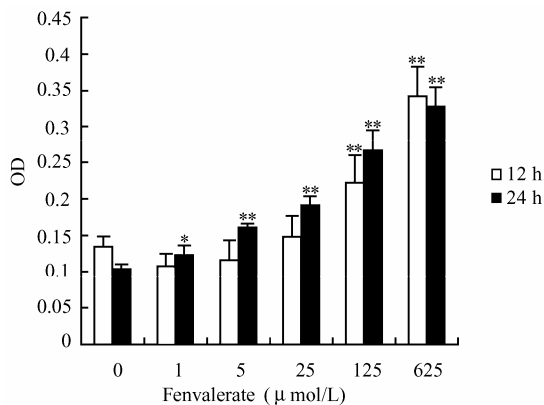


FIG. 1. Effects of fenvalerate on cell proliferation in rGCs. Cells were cultured for 12 h or 24 h in the presence of fenvalerate at various concentrations of (0, 1, 5, 25, 125, 625  $\mu\text{mol/L}$ ) in 96-well plates. Values are  $\bar{x} \pm s$  for six observations. Statistically significant differences are designated with (\*)  $P < 0.05$  or (\*\*)  $P < 0.01$ .

8-Bromo-cAMP (1 mmol/L) was used as a PKA activator. Compared with basal steroidogenesis, 8-Bromo-cAMP could significantly increase steroidogenesis, but no significant difference in fenvalerate-reduced steroid hormone levels was observed with or without the addition of 8-Bromo-cAMP (Fig. 4).

The water soluble cholesterol analog 22R-HC was used since it could readily diffuse to the P450 $\text{scc}$  enzyme located on the inner mitochondrial membrane, bypassing the need for StAR-mediated cholesterol transfer, the rate-limiting step in progesterone production. Fig. 5. shows that fenvalerate still reduced 22R-HC-driven progesterone production. However, as fenvalerate (25  $\mu\text{mol/L}$  and 125  $\mu\text{mol/L}$ ) only reduced FSH+22R-HC-driven progesterone production by 54.6% and 67.0%, respectively, after 24 h treatment, this shows that 22R-HC could partially reverse the inhibition of FSH-stimulated steroidogenesis.

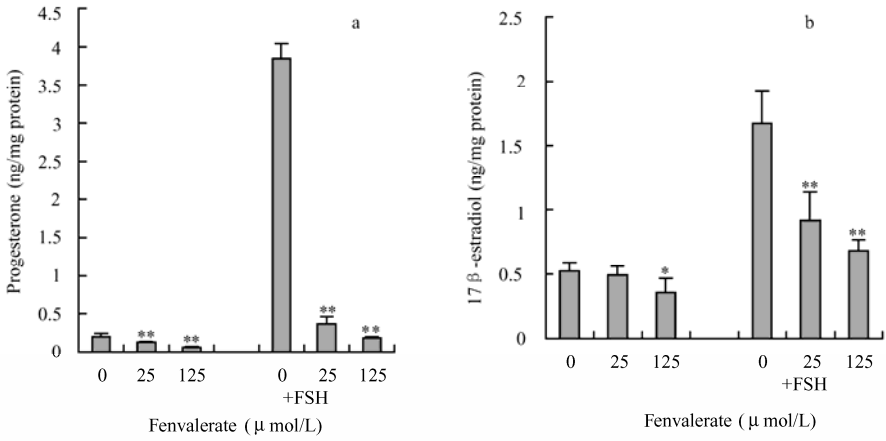


FIG. 3. Response profiles of FSH-stimulated steroidogenesis (2 mg/L) in rGCs cultured for 24 h in the presence or absence of fenvalerate as indicated on the histograms. Each data point is the  $\bar{x} \pm s$  of three replicates in a single experiment which was performed three times. Statistically significant differences are designated with (\*)  $P < 0.05$  or (\*\*)  $P < 0.01$ .

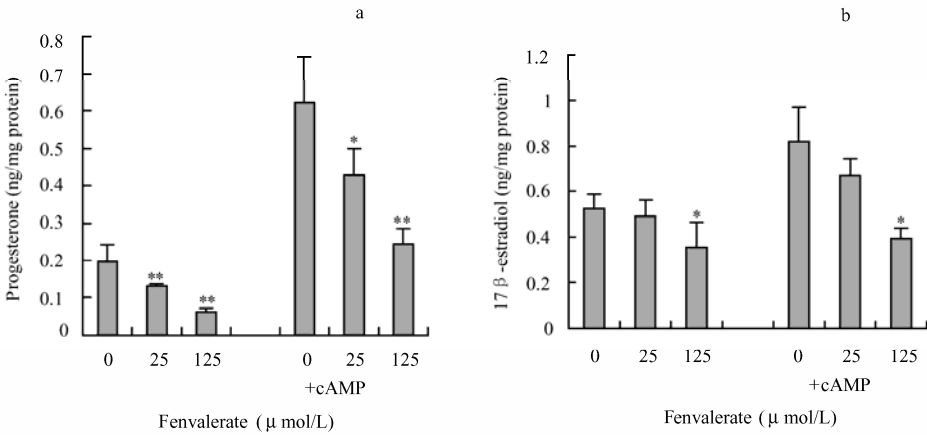


FIG. 4. Effects of fenvalerate on cAMP-stimulated steroidogenesis in rGCs. Cells cultured with fenvalerate at various concentrations in the presence or absence of 1 mmol/L 8-Bromo-cAMP for 24 h. Each data point is the  $\bar{x} \pm s$  deviation of three replicates in a single experiment which was performed three times. Statistically significant differences are designated with a (\*)  $P < 0.05$  or (\*\*)  $P < 0.01$ .

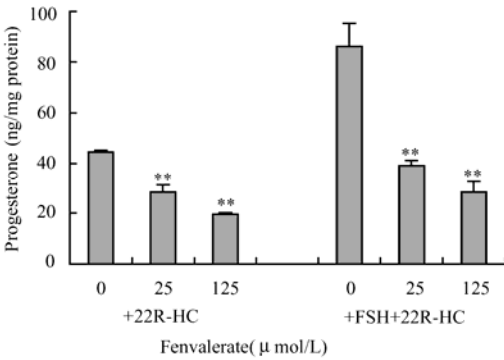


FIG. 5. Effects of fenvalerate on 22R-HC-stimulated steroidogenesis in rGCs. Cells cultured with fenvalerate at various concentrations in the presence of 25 μmol/L 22R-HC for 24 h. In some cases, 2 mg/L FSH was added to the cells. Each data point is the  $\bar{x} \pm s$  of three replicates in a single experiment which was performed three times. Statistically significant differences are designated with (\*\*)  $P < 0.01$ .

### Modulation of Steroidogenic Enzymes mRNA Expression in rGCs by Fenvalerate

To determine if the decrease in steroid hormone production might be due to reduced levels of steroidogenic enzymes, RT-PCR was used to detect the mRNA levels of P450scc, 3β-HSD and 17β-HSD in rGCs. As shown in Fig. 6, fenvalerate did not significantly change 3β-HSD mRNA level in rGCs (Fig. 6a). Surprisingly, although the progesterone production decreased (Fig. 2a), the mRNA expression level of P450scc significantly increased with increasing concentration of fenvalerate (Fig. 6b). Especially at the dose of 625 μmol/L, fenvalerate potentiated 3-fold increase in P450scc mRNA levels over the level observed in control.

Biosynthesis of E<sub>2</sub> in granulosa cell involves two steroidogenic enzymes, P450arom and 17β-HSD,

orchestrate FSH-dependent conversion of androgens into estrone ( $E_1$ ) and  $E_2$ , respectively. The changes in  $E_2$  production might be due to the inhibition or stimulation of these enzyme expressions. The previous study in our laboratory indicated that fenvalerate could not affect the activity of P450arom

(data not shown). So this study examined the mRNA level of 17 $\beta$ -HSD in rGCs treated with fenvalerate (Fig. 6c). Result shows that fenvalerate strongly inhibited 17 $\beta$ -HSD mRNA expression in a dose-response manner.

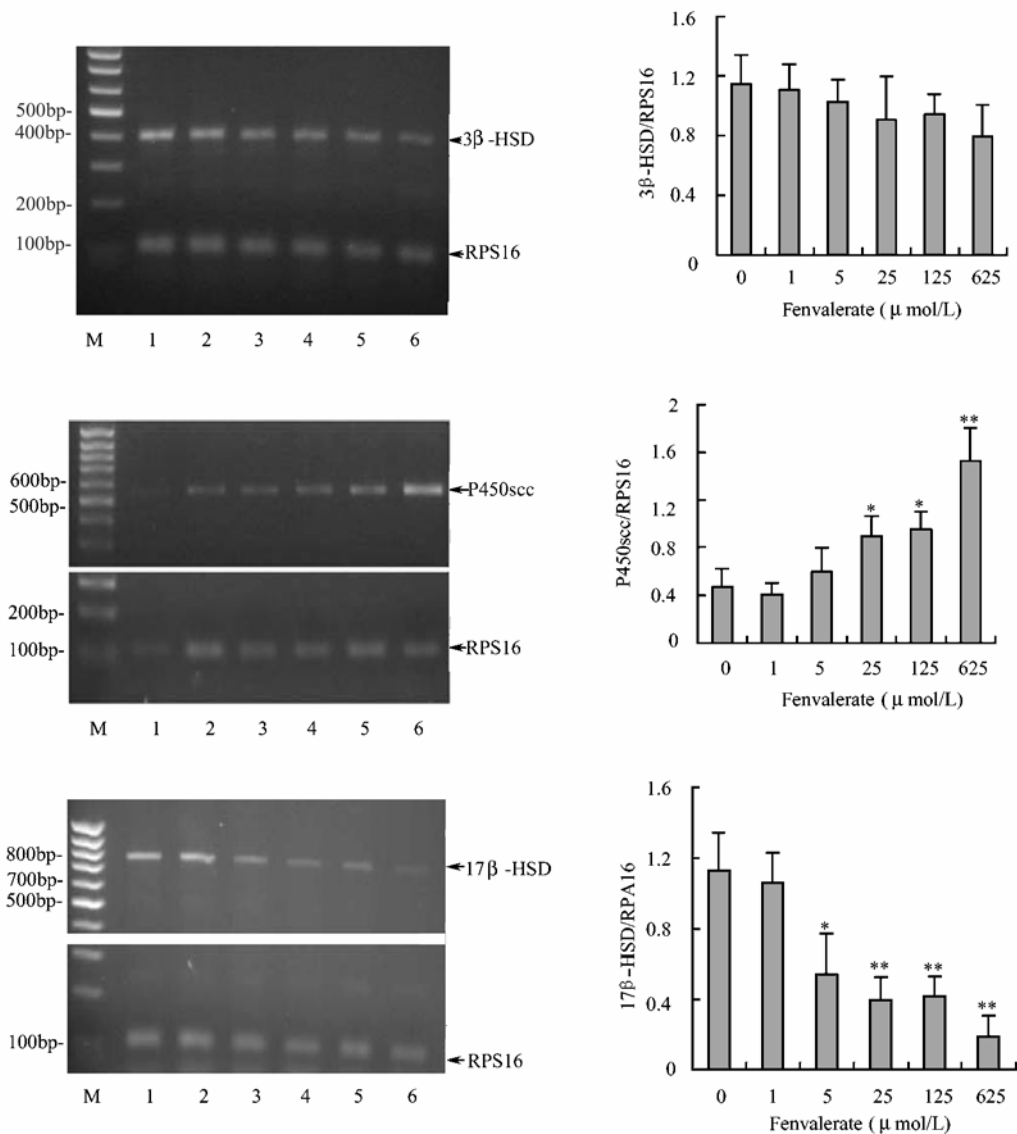


FIG. 6. Effects of fenvalerate on expression of 3 $\beta$ -HSD, P450scc and 17 $\beta$ -HSD genes in rGCs. Cells were cultured with fenvalerate at various concentrations in 6-well plates for 24 h. Total RNA was extracted and analyzed by RT-PCR as described in Materials and Methods. Left panels and photographs show the amplified PCR signals obtained for each set of primers. The levels of the RPS16 PCR products, which were not affected by hormonal treatments, served for quantitative normalization of the target gene products. The densitometric data are shown in the right panels. Values are the  $\bar{x} \pm s$  of three replicates in a single experiment which was performed three times. Statistically significant differences are designated with (\*\*)  $P < 0.01$ . M, Generuler™ 100bp DNA ladder; lanes 1-6, the various concentrations of fenvalerate, from 0 to 625  $\mu$ mol/L.

## DISCUSSION

Results from *in-vitro* experiments indicate the

effects of EDCs on the female reproductive system and suggest a hypothesis for their *in-vivo* action and their linkage with disease. Many of the EDCs have

been shown to competitively bind to the estrogen receptor and to initiate gene transcription in transfected cells. However, other mechanisms (e.g., hormone synthesis, transport, and metabolism) have been shown to be equally important. Granulosa cells have been found to play a significant role during the ovarian cycle and secretion of steroid hormones<sup>[18]</sup>. The present study used rGCs as model and showed that fenvalerate significantly decreased progesterone and 17 $\beta$ -estradiol production, although the mechanism remains not so clear. It indicated that fenvalerate might impair the balance of the endocrine system in women. However, further studies are necessary to determine how fenvalerate affects steroid hormone production.

Previous studies have shown that fenvalerate is an estrogen mimic as identified by MCF-7 cell proliferation test and pS2 gene expression test<sup>[5-6]</sup>. Estrogen receptors (ERs), notably ER $\beta$ , are present on granulosa cells<sup>[19-20]</sup> and estrogens are known to regulate granulosa cell function<sup>[21]</sup>. Furthermore, Nejaty *et al.*<sup>[22]</sup> reported that estrogen significantly inhibited basal progesterone production and forskolin (adenyl cyclase activator)-driven progesterone production at a high concentration of 10<sup>-5</sup> mol/L. The effects of fenvalerate might be explained as an estrogen mimic, for it could stimulate rGCs proliferation and significantly inhibit both basal and FSH-stimulated steroidogenesis.

In mammalian ovary, the growth and terminal differentiation of follicles require the coordinated expression of specific genes in granulosa cells and oocytes. The cells of follicles use an array of signaling pathways to interpret the external cues and ultimately to control the switching on and off of genes at the appropriate time during follicle growth and differentiation<sup>[11-25]</sup>. Three decades of research have established that cyclic nucleotide signaling plays a pivotal role in gonadotropin regulation of granulosa cells. FSH induces the sequential activation of stimulatory G proteins (G<sub>s</sub> proteins), adenylyl cyclase-directed generation of cAMP, and cAMP-dependent protein kinases (PKAs). PKA thereby promotes the dissociation and activation of the C subunit. The activated C either phosphorylates cytoplasmic substrates or controls gene expression. Thus, environmental toxicants disrupting or dysregulating the sites of such signaling cascades may lead to the blocking of steroidogenesis. The inhibition of fenvalerate to steroidogenesis in the presence of FSH was stronger than that in the absence of FSH in rGCs, indicating that this signal pathway was impaired by fenvalerate. The cAMP analogue, 8-Bromo-cAMP, was added to cells in order to activate PKA independent of adenylyl cyclase. However, suppression of fenvalerate to

steroidogenesis was not reversed. One explanation of these data is that fenvalerate interferes with the FSH-induced cAMP pathway behind the generation of cAMP. Recently, some studies disclosed that in granulosa cells FSH could produce signals via cascades in addition to that mediated by cAMP. For example, FSH could activate mitogen-activated protein kinase (MAPK) and tyrosine kinase pathways in granulosa cells<sup>[16-26]</sup>. It was shown that in a granulosa cell line MAPK activation inhibited steroidogenesis<sup>[27]</sup>. It is possible that fenvalerate may be functioning via MAPK to suppress steroidogenesis. Whether this is the case remains to be determined.

P450scc is part of the cholesterol side chain cleavage enzyme system (CSCC) which also includes adrenodoxin reductase and adrenodoxin. Its function of converting cholesterol to pregnenolone also is the rate-limiting step in steroidogenesis, the StAR protein delivers cholesterol from the outer to the inner mitochondrial membrane. The elevation of P450scc mRNA level can lead to the increase of progesterone. An interesting observation in the present study was that fenvalerate increased the level of P450scc mRNA while decreased the progesterone production. But the decrease could have been explained by a decrease in enzyme activity in addition to a general reduction in protein synthesis. Further studies are necessary to determine whether and how fenvalerate affects P450scc activity. In addition, fenvalerate may affect other sites in granulosa cell steroidogenesis, suggesting that the increase of P450scc mRNA level has little physiological significance.

Some studies reported that pesticides, such as dimethoate and lindane could inhibit steroidogenesis in mouse MA-10 Leydig tumor cells primarily by blocking transcription of the StAR gene, indicating that StAR may be an important target for environmental pollutants<sup>[23-24]</sup>. In steroidogenesis steroid production is very sensitive to alterations in StAR protein expression. This study used 22R-HC as substrate of P450scc enzyme, bypassing the need for StAR. Results showed that 22R-HC alone dramatically increased progesterone production. Although fenvalerate still decreased 22R-HC-driven or FSH+22R-HC-stimulated progesterone production, the inhibition of FSH-stimulated steroidogenesis could partially be reversed. Perhaps fenvalerate also disrupted StAR protein expression that partially accounted for the reduction of steroidogenesis. However, the mRNA expression, protein synthesis and phosphorylation status of StAR protein and other proteins that are crucial for steroidogenesis were not directly assessed, changes in other sites could not be excluded and further studies are needed.

17 $\beta$ -HSD plays an essential role in steroidogenesis. 17 $\beta$ -HSD type I catalyzes the final steps in

estrogen biosynthesis. This study showed that fenvalerate could decrease the 17 $\beta$ -HSD mRNA expression level, consisting of the decrease of E<sub>2</sub> production. At all doses except 1  $\mu$ mol/L fenvalerate significantly suppressed 17 $\beta$ -HSD mRNA level while only at 125  $\mu$ mol/L it began to inhibit E<sub>2</sub> production. That is, the impairment of 17 $\beta$ -HSD mRNA expression appeared prior to the decrease of estrogen production. Perhaps it was due to 17 $\beta$ -HSD enzyme which is chronically regulated and has a long half-life, a more protracted exposure to low dose fenvalerate may also reduce the levels of this enzyme and may ultimately contribute to the decline in steroidogenic capacity of cells. In ovarian granulosa cells, 17 $\beta$ -HSD type I expression is strictly regulated by pituitary gonadotrophins, steroid hormones and growth factors and this regulation is a nonclassic cAMP-dependent mechanism<sup>[28-29]</sup>. Its role in the mechanism of down-regulating 17 $\beta$ -HSD gene by fenvalerate remains to be determined.

In conclusion, fenvalerate inhibits both P<sub>4</sub> and E<sub>2</sub> production in rGCs. The mechanisms may involve the effects on the steroidogenesis signaling cascades and/or steroidogenic enzymes' activity. While a cause-and-effect relationship between the presence of pesticides and reproductive dysfunction via endocrine disruption remains to be established, the potential for pesticides to disrupt reproductive function is real. It indicates that the potential health implication of EDCs deserves serious consideration. Thus, these findings underscore the need for further studies to assess the effects of pesticides and other environmental pollutants on wildlife and humans in their natural habitats.

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