Journal of Food and Nutrition Research, 2018, Vol. 6, No. 2, 116-123
Available online at http://pubs.sciepub.com/jfnr/6/2/8

DOI:10.12691/jfnr-6-2-8

Effects of Herbal Mixture Extract on Menopausal Hot Flashes and Pharmacokinetics in Ovariectomized Rat Models

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Abstract This study was conducted to evaluate the effects of *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turcz., and *Angelica gigas* Nakai extract (CPAE) on the reduction of tail skin temperature (TST) in ovariectomized (OVX) rats. To evaluate TST reduction in ovariectomized rats, 125, 250, and 1000 mg/kg CPAE was administered to rats for 1 week. The measurement of TST after the induction of artificial stress revealed a significant temperature reduction effect in the CPAE administration group (p<0.05). The TST induced by the injection of yohimbine, to induce hot flashes, was found to decrease as the administered dose of CPAE increased from 10 min to 20min (125 and 250 mg/kg/day, p<0.05; 1000 mg/kg/day, p<0.01). In addition, in a drug interaction test between tamoxifen, an anti-estrogen drug, and CPAE, no significant difference was found between the pharmacokinetic (PK) profiles after the administration of tamoxifen only and the combination of tamoxifen and CPAE. We also found that CPAE did not affect CYP2d4 and CYP3a2, which affect tamoxifen metabolism, in a subsequent experiment on liver tissues extracted during the drug interaction test. In this study, we found that CPAE inhibited temperature increase on the tail skin of OVX rats, and therefore is effective in the improvement of hot flashes. CPAE may be potentially used for the improvement of hot flashes induced by the administration of tamoxifen.

Keywords: *Cynanchum wilfordii, Cytochrome P450, Hot Flash, Tail Skin Temperature, Tamoxifen*

Cite This Article: Gyuok Lee, Jaeyong Kim, Huwan Kang, SungYoon Park, Junkee Hong, Joohyun Oh, Jimin Kim, Chansung Park, Yongwook Lee, and Chul-yung Choi, "Effects of Herbal Mixture Extract on Menopausal Hot Flashes and Pharmacokinetics in Ovariectomized Rat Models." *Journal of Food and Nutrition Research*, vol. 6, no. 2 (2018): 116-123. doi: 10.12691/jfnr-6-2-8.

1. Introduction

Owing to the increased human life expectancy that has resulted from socioeconomic advancements and developments in medical technology, the post-menopausal period now accounts for over one-third of a woman's lifespan. Most women in Korea experience menopause at 51-52 years of age. The minimum and maximum ages at menopause are 39 and 60 years, respectively; the mean age at menopause is 48 years [1]. A loss of ovarian function occurs over a 5-10-year period before and after menopause. As estrogen secretion is reduced to the point at which it does not meet the body's demand, the reproductive system becomes non-reproductive [2,3]. The menopause causes major changes in the body, including changes in the hormones released from the ovaries. This can lead to an irregular menstrual cycle, reduced vaginal discharge, unstable autonomic nerve function, and consequently, hot flashes and sweating [4]. Most women start to experience these symptoms 3-4 years before menopause. As the changes in ovarian function typically occur in the year after menopause, these changes occur at between 39 and 51 years of age in 95% of women, and the menopausal period lasts 5 years on average. Thus, menopause occurs over a period of 2-8 years in 95% of women [5].

Hot flashes occur predominantly in the early evening, usually when a person is anxious or excited, has consumed spicy food or hot drinks, is under undue stress, or when the weather is hot. Hot flashes are of varying duration, from a few seconds to an hour; commonly, they last between one and five minutes [6]. The manifestation of a hot flash is a typical symptom of estrogen deficiency. It results from disharmony in the autonomic nervous system, which causes the peripheral blood vessels to suddenly dilate and contract. Most menopausal women experience this symptom to a certain extent. In general, a hot flash is characterized by the spreading of the sensation of heat from the chest, neck, and face to other parts of the body, accompanied by red patches, perspiration, palpitations, anxiety, and insomnia. The increased body temperature induces perspiration through a mechanism that is yet to be established, but may be associated with decreased estrogen levels [7,8].

Hormone replacement therapy (HRT), in which estrogen is administered with or without progesterone, is a common treatment for menopause symptoms, such as hot

flashes, which result from decreased estrogen levels. However, concerns about the safety and benefits of HRT have arisen owing to reports of cardiovascular diseases, stroke, thromboembolism, breast cancer, and dementia [9]. As an alternative to HRT, selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs), which are also used as antidepressants, have been introduced to treat hot flashes; various phytoestrogens and vitamin E have also been used in place of HRT [10].

Tamoxifen, the most widely used drug for the treatment of breast cancer, inhibits the function of estrogens [11]. Hot flashes occur in 60-70% of women who use tamoxifen [12,13,14]. In a controlled experiment that involved menopausal women with breast cancer who were prescribed tamoxifen, 67% of patients who ingested tamoxifen had vascular disorder, which indicated that tamoxifen use was associated with hot flashes [15,16]. However, as SSRIs can interfere with the metabolism of tamoxifen [17], it is difficult to prescribe them to patients with breast cancer who are currently using tamoxifen and experience hot flashes. Therefore, it is necessary to develop safe and effective drugs or functional ingredients that can be prescribed to women who suffer from menopausal symptoms such as hot flashes.

A previous study reported that CPAE (*Cynanchum wilfordii* (CW), *Phlomis umbrosa* (PU), and *Angelica gigas* (AG) extracts, Brand name: EstroG-100®) relieved various symptoms associated with menopause such as hot flashes [18]. In addition, it was verified to be an effective and safe functional food for perimenopausal, menopausal, and postmenopausal women by clinical study [19]. However, the mechanism by which CPAE relieves hot flashes is currently unknown. Thus, in this study, the tail skin temperature (TST) and rectal temperature (RT) of ovariectomized rat models, which are widely used in research on osteoporosis and cardiovascular diseases in women, were assessed by using a popular technique to induce menopause [20]. The technique comprised of a reduction in estrogen levels, induction of stress through the application of physical pressure, and increase in body temperature through the injection of yohimbine. In addition, to investigate the co-prescription of CPAE and tamoxifen, the serum level of tamoxifen and the expression of CYP isozymes in liver tissues were measured to analyze the drug-drug interactions.

2. Material and methods

2.1. Samples and Preparation

To prepare CPAE, CW and PU were purchased from Jirisan Farming Association (Sancheong, Gyeongsangnam-do, Korea) and Baocheng Chinese Herbal Med Co., Ltd (Hunan, China), respectively, in July 2015, and AG was provided by Yeongdong Herb Medicine Farming Association (Jecheon, Chungcheongbuk-do, Korea) in October 2015. CW, PU, and AG were mixed at the ratio of 1:1:1.08, extracted with distilled water (1:8, w/v) by boiling under reflux for 8 h, cooled to room temperature (22 ± 2 ℃) for 30 min, and filtered through Whatman #4 filter paper (Whatman, Maidstone, MA, USA). The filtrate was subsequently evaporated by using a rotary evaporator (R-215, Buchi, Switzerland) under reduced pressure until 40 °brix and lyophilized with a freeze drier (DW-86L728, Haier, China).

2.2. Animals and Treatments

Ten-week-old female Sprague-Dawley rats (Samtako, Osan, Korea), were housed in well-ventilated cages at room temperature (22 ± 2 ℃), 50.0% ± 15% relative humidity, and a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of 1 week prior to the experiment. Then, one group received a bilateral ovariectomy using a dorsal approach (OVX; n=50) and the other group underwent a sham operation (sham; n=7) as a control. The OVX rats were randomly divided into five groups: OVX group (OVX/vehicle; n=10), E2 treatment group (OVX/E2 91 μg/kg body weight/day; n=10), and three CPAE treatment groups (OVX/CPAE 125, 250, or 1000 mg/kg body weight/day; n=10). Starting at 1 week after surgery, the test compounds or distilled water was orally administered to rats at 0.05 mL/kg body weight once per day for 7 days. Distilled water (10 mg/kg) was administered to the sham-operated rats for 7 days following the same schedule as the control. The procedures used in the present study complied with the animal care guidelines in the "Principles of Laboratory Animal Care" (NIH publication no.23-85, 1996). All experiments involving animals were approved by the Animal Ethical Committee of Jeollanamdo Institute for Natural Resources Research (JINR1708).

2.3. Measurement of TST and RT

Stress was induced by the application of artificial physical pressure. To induce stress by using yohimbine, 3 mg/kg yohimbine was injected 30 min after the last sample administration. The TST was measured 10 min before injection yohimbine, and the TST and RT were measured at 10-minute intervals for 1 h after the first injection.

The rats were restrained in a holder in a conscious state and the TST was measured on the dorsal surface of the tail, approximately 2 cm from the fur line, by using an infrared thermometer (AMIR 7210, Ahlborn Mess- & Regeltechnik GmbH, Holzkirchen, Germany). Before testing, all animals were settled in the laboratory room for 15 min. The environmental temperature was 25 ℃. The TST measurements over the 1 h period were used to calculate the change in TST for each 10 min measurement compared with the mean TST at 0 min (ΔTST).

$$\Delta\text{TST} = (\text{TST in each 10 min block}) - (\text{TST at 0 min}).$$

The values were expressed as the mean ± standard error of the mean (SEM).

RT was measured by using a microprobe thermometer (BAT-12, Physitemp, Clifton, NJ, USA), which was inserted 5 cm into the rectum. The probe was dipped into glycerol before insertion and held in the rectum for approximately 20 s. The measurements were recorded in duplicate. Temperature recordings were performed by the same person that handled the animals prior to the experiment.

$$\Delta RT = (RT \text{ in each 10 min block}) - (RT \text{ at 0 min}).$$

The values were expressed as the mean ± SEM.

2.4. Experimental Procedure of Pharmacokinetics

Six-week-old female Sprague-Dawley rats were procured from KOATECH (Pyeongtaek, Korea) and acclimatized for 1 week prior to the experiment. The rats underwent bilateral ovariectomy and were permitted a 1-week recovery period. Tamoxifen (Sigma Aldrich Ltd, USA) was dissolved in 0.5% carboxymethylcellulose solution. The CPAE dose was selected as 200 mg/kg. The rats were divided into two groups (n = 4, each): group I contained ovariectomized rats administered a single oral dose of tamoxifen (200 mg/kg); group II contained ovariectomized rats, who were administered a CPAE (200 mg/kg) pretreatment for 1 h followed the administration of tamoxifen (200 mg/kg). The blood samples were collected in EDTA-2K in a Vacutainer tube (BD, USA) from both groups at 0.1, 0.5, 1, 2, 4, 8, 24, 48, 72, and 96 h post-dose; the plasma samples were obtained by centrifuging the blood at 13000 rpm for 15 min at 4 ℃ and stored frozen at –70 ℃±10 ℃ until bioanalysis. The rat livers were separated and frozen at –70 ℃±10 ℃ for mRNA extraction.

2.5. Preparation of Plasma Sample

The generated study samples were prepared by the validated HPLC method [21]. In brief, 150 μL acetonitrile was added to 150 μL of the plasma samples, vortex mixed for 5 min (Vortex-genie2, USA), and centrifuged for 10 min at 1350 rpm (Wisespin, South Korea). The supernatant was collected and transferred to new tube. The pellet was subjected to the extraction procedure a further two times. The supernatant was combined and evaporated by N_2 gas at 60 ℃. The concentrates were completely dissolved in 100 μL acetonitrile, mixed for 10 min on a vortex mixer, and centrifuged for 10 min at 1350 rpm. Finally, 20 μL of this solution was removed for HPLC analysis.

2.6. Equipment and Chromatographic Conditions

The generated study samples were analyzed for tamoxifen by the validated HPLC method published by Marina et al. [22]. The HPLC system consisted of a photodiode array detector and an Agilent HPLC 1260 (Agilent Technology, USA). The separation was performed on a Capcell Pak UG120 C18 column (250 mm × 4.6 mm I.D., particle diameter 5 μm) from Shiseido Co., LTD. (Tokyo, Japan). The mobile phase was a mixture of triethylammonium phosphate buffer 5 mM pH 3.3 (A) and acetonitrile (B), used in the following gradient elution (v/v); 0–9 min, 35–50% B; 9–17 min, 50% B; 17–19 min, 50–100% B. Subsequently, the column was washed and reconditioned. The flow rate was set at 1.0 mL/min, an injection volume of 20 μL was used, and the temperature was set at 35 ℃. The total run time was 45 min. The spectra were acquired between 200 and 380 nm, and the quantitation wavelength was 200 nm.

2.7. Total RNA Extraction and RT-PCR

Total RNA was extracted from rat liver tissues by using TRIzol reagent in accordance with the manufacturer's instructions. The quality and quantity of the isolated RNA was assessed at the 260/280 nm absorbance ratio 1.8–2.0 using Synergy H1 (BioTek, USA). RNA was transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (AB, USA) and oligo $d(T)_{18}$ primers in accordance with the manufacturer's instructions. The following conditions and primers were used for the PCR: CYP1a1, sense 5'-CATTTGAGAAGGGCCACATC-3' and antisense 5'-CCAATCACTGTGTCTAACTCCTCC-3'; CYP2b2, sense 5'-CTTTGCTGGCACTGAGACCG-3' and antisense 5'-ATCAGTGTATGGCATTTTGGTACGA-3'; CYP2c11, sense 5'-CTGCTGCTGCTGAAACACGTG-3' and antisense 5'-GGATGACAGCGATACTATCAC-3'; CYP2d4, sense 5'-GACCAGTCGGGCTTTGGACCAC-3' and antisense 5'-CGAAGGCCTTCTTTCCAGAG-3'; CYP2e1, sense 5'-CTCCTCGTCATATCCATCTG-3' and antisense 5'-GCAGCCAATCAgAAATGTGG-3'; CYP3a2, sense 5'- TACTACAAGGGCTTAGGGAG-3' and antisense 5'-CTTGCCTGTCTCCGCCTCTT-3'. Gapdh was amplified as a control gene using the following primers: sense 5'-TCGTCTCATAGACAAGATGG-3' and antisense 5'-GTAGTTGAGGTCAATGAAGGG-3', and the gene expression changes were normalized to Gapdh expression. The resulting amplification products were analyzed by agarose gel electrophoresis and image analysis.

2.8. Statistical Analysis

All values were presented as the mean ± SEM. The statistical significance was evaluated by unpaired Student's t-test. Statistical significance was accepted for values of $p<0.05$.

3. Result

3.1. Investigation of Stress-induced OVX Rat Models

The rat models in the control group (ovariectomized and TST increased by the application of physical pressure) showed a drastic temperature increase after 20 min, an increase of 4.30 ℃±1.12 ℃ after 50 min, and 4.48 ℃±0.81 ℃ after 60 min. In contrast, an overall suppression of temperature increase was observed in the OVX rats that were administered CPAE. In the 125 mg/kg CPAE group, temperature increases of 0.70 ℃±0.96 ℃ and 0.62 ℃±1.00 ℃ were observed after 50 and 60 min, respectively; in the 250 mg/kg CPAE group, temperature increases of 0.80 ℃±0.09 ℃, 1.04 ℃±0.41 ℃, and 1.20 ℃±0.58 ℃ were observed after 40, 50, and 60 min, respectively; and the 1000 mg/kg CPAE group showed a temperature increase of 1.23 ℃±0.78 ℃ after 60 min. Therefore, it was concluded that CPAE significantly suppressed the temperature increase ($p<0.05$), and significant suppression was observed from 40 min onwards in the 250 mg/kg CPAE group. A similar result was observed in the positive control group, in which the rats were administered E2 (Figure 1).

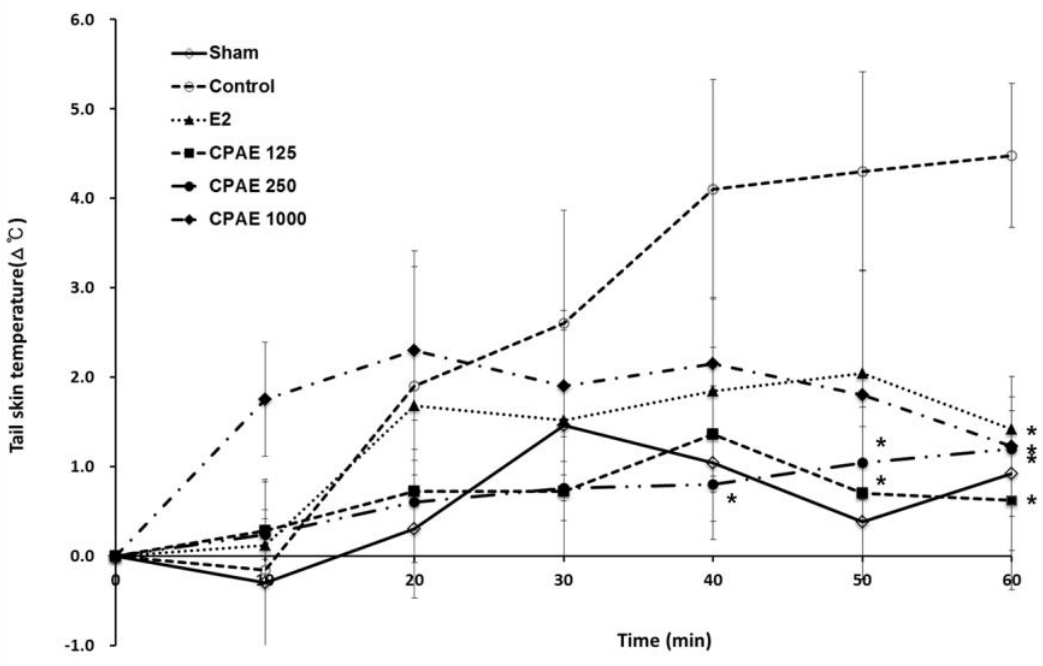

Figure 1. The effects of CPAE on stress-induced changes in tail skin temperature in female rats. The data are presented as the mean±S.E.M of 6-8 animals. * $p<0.05$, significantly different from the OVX control group. Statistical significance was analyzed by using the unpaired Student's *t*-test

3.2. Investigation of Yohimbine-induced OVX Rat Models

TST and RT were observed after 3 mg/kg yohimbine injection. At 10 and 20 min after the injection, the highest increase in TST was observed in the experimental and control groups. After 10 min, the TST was increased by 3.93 ℃±0.05 ℃ in the control group, but was only increased by 1.75 ℃±0.72 ℃ ($p<0.05$), 1.55 ℃±0.44 ℃ ($p<0.01$), and 1.23 ℃±0.32 ℃ ($p<0.01$) in the CPAE 125, 250, and 1000 mg/kg groups, respectively, which indicated that the temperature increase was significantly suppressed by all CPAE concentrations. In addition, CPAE suppressed the temperature increase more effectively than E2. A similar result was observed in the positive control group (Figure 2). The RT was significantly lower after the yohimbine injection than before. Moreover, no significant difference in the changes in the RT was observed between the control and the CPAE groups (Figure 3).

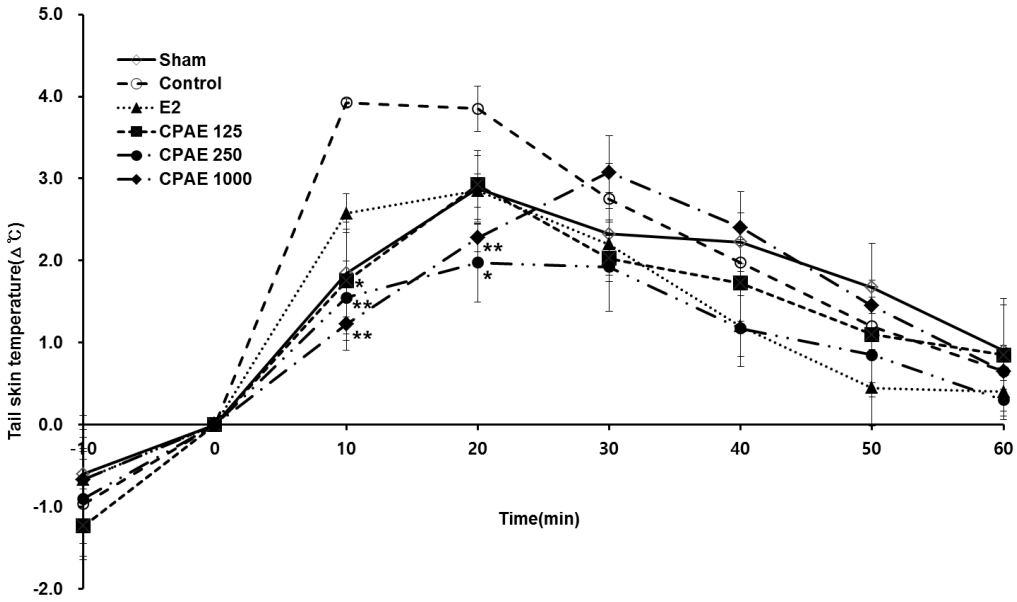

Figure 2. The effects of CPAE on yohimbine-induced changes in tail skin temperature in female rats. The data are presented as the mean ± S.E.M of 6-8 animals. *$p<0.05$, **$p<0.01$, significantly different from the OVX control group. Statistical significance was analyzed by using the unpaired Student's *t*-test

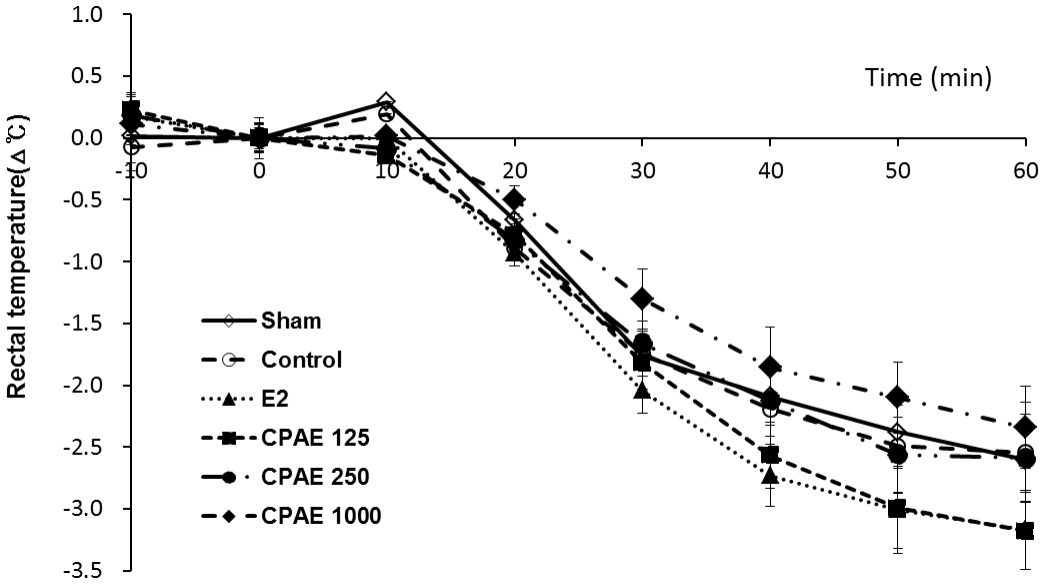

Figure 3. The effects of CPAE on yohimbine-induced changes in the rectal temperature of female rats

3.3. Assessment of Uterine Hypertrophy Induced by CPAE

To investigate whether the uterine weight of CPAE-treated rats was increased, the uterine weight was measured, and the comparison of the uterine weights revealed a significant reduction in the OVX control group (0.05±0.002 g/100 g B.W.) compared with the sham group (0.29±0.03 g/100 g B.W.), with no significant increase from CPAE administration (Figure 4). However, a significant increase in the uterine weight was observed in the E2 group (p<0.05). No significant differences in the weight of the liver, kidney, adrenal cortex, and pituitary gland were observed (data not shown).

These two animal experiments demonstrated CPAE relieved the sensation of heat induced by stress and yohimbine. Thus, CPAE may relieve the sensation of heat caused by a hot flash, without causing a direct effect on the uterus.

3.4. Pharmacokinetics of Tamoxifen in the Presence of CPAE

The mean plasma concentration profiles of tamoxifen with oral administration of tamoxifen (200 mg/kg) alone or in combination with CPAE (tamoxifen+CPAE, CPAE 200 mg/kg) in OVX rats are shown in Figure 5.

The tamoxifen pharmacokinetic parameters were calculated based on these parameters, AUC was 161.247 ng·h/mL for tamoxifen and 153.943 ng·h/mL for tamoxifen+CPAE. The Cmax values were 2.404 ng/mL and 2.447 ng/mL, respectively; the Tmax was 4 h. No significant differences were observed in these parameters.

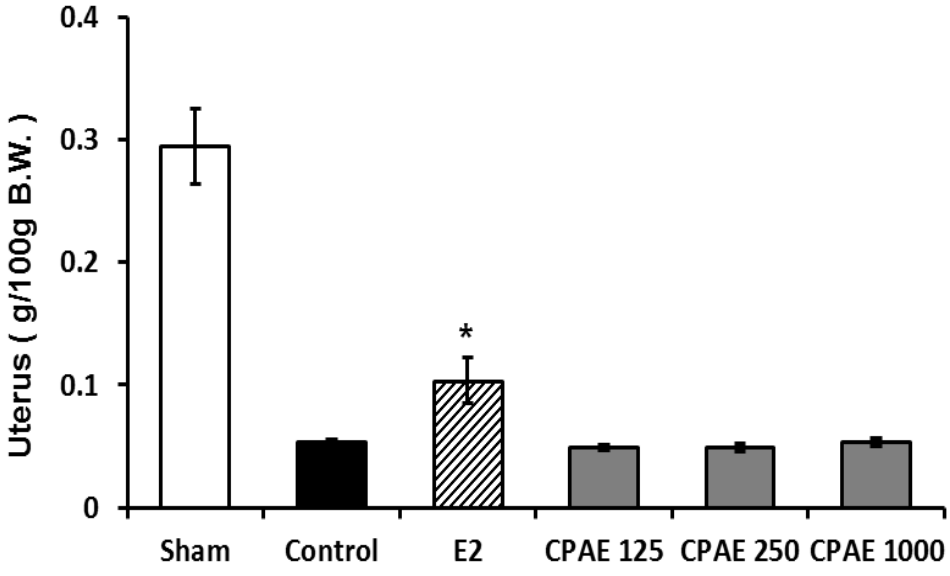

Figure 4. The effects of CPAE on uterus index in ovariectomized rats. *p<0.05, significantly different from the OVX control group. Statistical significance was tested by using the unpaired Student's t-test

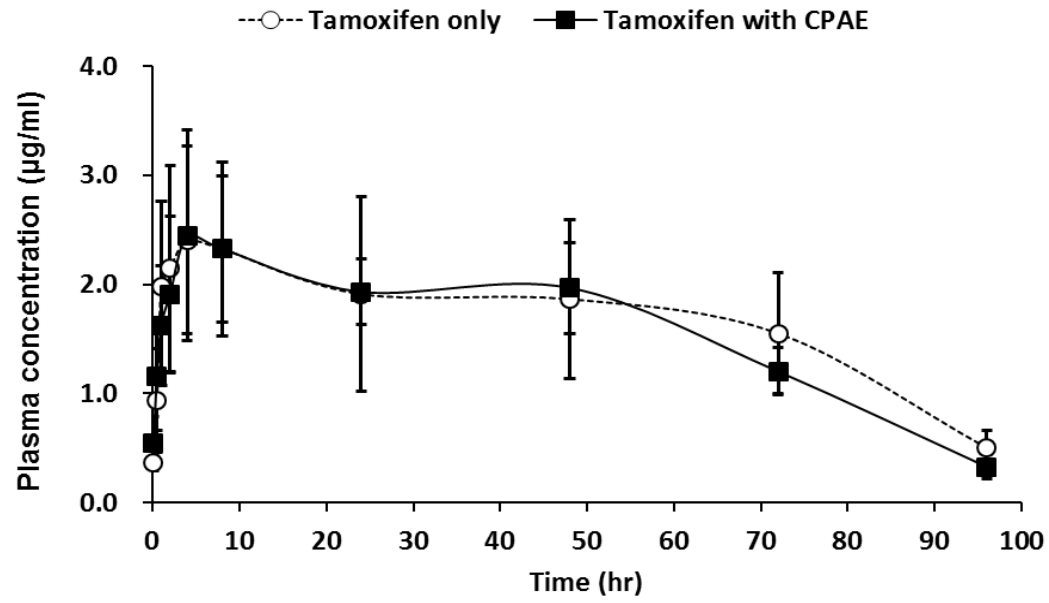

Figure 5. The plasma concentration profiles of tamoxifen after the administration of tamoxifen, in the presence or absence of CPAE, to OVX rats. (n=4)

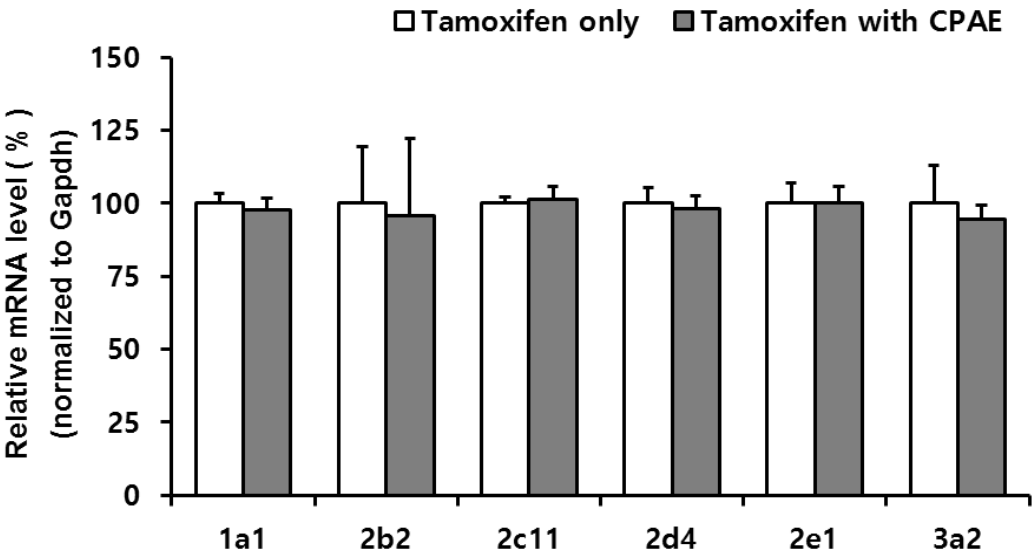

Figure 6. The effects of CPAE on the expression levels of the cytochrome P450 enzymes 1a1, 2b2, 2c11, 2d4, 2e1, and 3a2 compared with the rats administered tamoxifen only

3.5. Expression of CYP Isozymes after CPAE Ingestion

The effects of CPAE on the gene expression of CYP450 isozymes in the liver tissues of OVX rats that were administered tamoxifen or tamoxifen+CPAE are shown in Figure 6. No significant changes in the expression of the CYP450 isoenzymes, CYP1a1, CYP2b2, CYP2c11, CYP2d4, CYP2e1, and CYP3a2, were observed. No differences in the expression of CYP2D6 (rat 2d4) and CYP3A4 (rat 3a2), which are the main metabolic enzymes of tamoxifen, were observed in the tamoxifen or tamoxifen + CPAE groups. These results indicated that CPAE did not affect the metabolism of tamoxifen in the liver into the active compounds such as 4-hydroxytamoxifen and N-desmethyl-4'-hydroxytamoxifen.

4. Discussion

In OVX rats, the level of estrogen hormones drastically decreased and skin temperature increased. As their bodies are entirely covered in hair, rats dissipate heat through their tails. In estrogen-deficient rats, the dilation of blood vessels leads to increased tail skin temperature (TST). A similar mechanism occurs in menopausal women to give rise to hot flashes; consequently, various studies have used this technique to assess hot flashes [23,24,25,26]. Experiments on OVX rats make use of various triggers. Previous studies have found that OVX rats increased TST by the induction of stress with physical exercise [27]. Numerous studies have also reported that yohimbine administration causes hot flashes [28,29]. Yohimbine, an α_2-adrenoceptor antagonist, is known to cause hot flashes

through the reduction of the thermoneutral zone, which affects temperature homeostasis [30,31]. FG-7142, calcitonin gene-related peptide, and neurokinin B have also been used to trigger hot flashes [32,33,34].

Our previous study reported that C. wilfordii inhibited the elevation of skin temperature in OVX rats and did not affect plasma estrogen activity and uterine weight [35]. In this study, CPAE (*Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turcz., and *Angelica gigas* Nakai extract) effectively relieved hot flash, a symptom of menopause, in OVX rat models. Improvements of hot flashes were measured after the administration of CPAE for 1 week in OVX rat models. Stress was induced by the application of physical pressure and the TST was measured for up to 60 min. A significant suppression of the increase in TST was observed in all CPAE-treated groups relative to the control and E2 groups at 60min. Next, TST and RT were measured after yohimbine injection. A significant suppression of TST was observed in all CPAE-treated groups relative to the control and E2 group at 10 min. However, no significant difference in RT was observed between all groups. These results suggested that CPAE is a useful functional ingredient for the relief of hot flashes.

Tamoxifen competitively binds estrogen receptors in tumors and other tissues to inhibit DNA synthesis, estrogen activity, and cell division. For this reason, it is injected into patients with estrogen-related tumors such as breast cancer. However, drugs with anti-estrogen activity, such as tamoxifen, can cause estrogen deficiency, which leads to symptoms such as hot flashes; therefore, they must be used in combination with other drugs or health functional foods to relieve these symptoms. Inside the human body, tamoxifen is predominantly metabolized into 4-hydroxytamoxifen and N-desmethyl-4'-hydroxytamoxifen. 4-Hydroxytamoxifen is an active metabolite that is 30 times more active than tamoxifen. About 7% of metabolism of tamoxifen is metabolized by CYP450 2D6 (rat 2d4), and about 92% of metabolism of tamoxifen is metabolized by CYP450 3A4 (rat 3a2) [36]. In other words, CYP450 2D6 and 3A4 are crucial for the metabolism of tamoxifen, and when the metabolism of tamoxifen is disrupted and metabolites are not produced correctly, tamoxifen could have its defined efficacy. In this study, no changes in the plasma tamoxifen level were observed in the tamoxifen+CPAE group compared with the tamoxifen group. In addition, CPAE did not affect the expression of CYP450 2D6 and 3A4, which demonstrated that CPAE did not affect the metabolic processes of tamoxifen being converted into its active metabolites. In conclusion, CPAE relieved hot flash without affecting estrogen levels or the metabolism of tamoxifen, and may therefore be suitable for development into a functional food or drug for the relief of hot flash. In future, we aim to investigate the pharmacokinetic profile through human clinical trial of tamoxifen administered with or without CPAE and assess the ability of CPAE to relieve adverse events caused by anti-estrogen drugs, including tamoxifen.

Disclosure

The authors declare no conflict of interest.

Acknowledgements

This research was supported by the Support Program For Creative Industry Institutes(Commercial Bio-technology Sophistication Platform Construction Program, R0003950) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea)

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