Oncology and Translational Medicine
DOI 10.1007/s10330-021-0510-0
August 2022, Vol. 8, No. 4, P191–P195

ORIGINAL ARTICLE

Association of 2-methoxyestradiol levels with the occurrence and development of endometrial cancer in humans*

Huanhuan Zhao[1], Junyu Li[1], Yan Liu[2], Li Li[1] (✉)

[1] Department of Obstetrics and Gynecology, The Forth Hospital, Hebei Medical University, Shijiazhuang 050011, China
[2] Department of Pharmacy, Hebei Medical University, Shijiazhuang 050017, China

Abstract

Objective The aim of the study was to determine the association of urinary levels of estradiol (E_2) and 2-methoxyestradiol (2-$MeOE_2$) with the occurrence and development of endometrial cancer.

Methods In this case-control study, 24-h urine specimens were collected from 28 postmenopausal patients with endometrial cancer and 28 postmenopausal healthy female controls. The concentration of 2-$MeOE_2$ was determined using liquid chromatography-mass spectrometry with hollow fiber liquid-phase microextraction. The concentration of E_2 was determined using an enzyme-linked immunosorbent assay.

Results Estrogen levels were different between the patients with endometrial cancer and controls. The relative quantity of E_2 in the case group was higher than that in the control group ($P < 0.05$), whereas that of 2-$MeOE_2$ was lower in the case group than that in the control group ($P < 0.05$). The ratio of E_2-to-2-$MeOE_2$ in the case group was significantly higher than that in the control group ($P < 0.05$).

Conclusion The results of this study indicate an imbalance of estrogen metabolites in endometrial carcinogenesis. Reduced 2-$MeOE_2$ levels and elevated E_2-to-2-$MeOE_2$ ratio may be used as potential biomarkers for the risk assessment of estrogen-induced endometrial cancer.

Received: 23 July 2021
Revised: 6 April 2022
Accepted: 21 June 2022

Key words: endometrial cancer; 2-methoxyestradiol (2-$MeOE_2$); estradiol (E_2); urine; high-performance liquid chromatography-mass spectrometry (HPLC-MS)

Endometrial cancer is estrogen-dependent. It is believed that exposure to estrogens in the absence of progesterone increases the risk of developing endometrial cancer [1]. Estradiol (E_2) is a sex hormone with strong biological activity, valuable in diagnosing and discriminating endocrine and gynecologic diseases. Estrogen metabolites are closely related to the occurrence and development of tumors [2]. An important metabolite of E_2, 2-methoxyestradiol (2-$MeOE_2$), is produced by cytochrome P450 1B1 and catechol-O-methyltransferase (COMT) [3]. During the last decade, 2-$MeOE_2$ has received considerable attention owing to its anticancer activity. Phase I and II clinical trials have revealed that orally administered 2-$MeOE_2$ is well tolerated by patients with grade 2 and grade 3 toxicities [4–7]. It is believed that 2-$MeOE_2$ acts directly on tumor growth by reducing cell proliferation, inducing apoptosis, and inhibiting angiogenesis [8]. A previous study has shown that 2-$MeOE_2$ inhibited the growth of endometrial cancer cells by inducing apoptosis and cell cycle arrest [9]. Therefore, it would be interesting to elucidate the mechanism of action of 2-$MeOE_2$.

Quantitative measurement of endogenous 2-$MeOE_2$ may play an important role in elucidating the mechanism underlying endometrial carcinogenesis; however, the low content of 2-$MeOE_2$ in the human body limits its measurement. Current methods for measuring endogenous catechol estrogens involve radioimmunoassay [10], enzyme immunoassay [11], high-performance liquid chromatography (HPLC) [12], liquid chromatography coupled with mass spectrometry [13], and gas chromatography-mass spectrometry [14]; however,

✉ Correspondence to: Li Li. Email: lily_lucky1@163.com
* Supported by the Hebei Province Medical Science Research Key Project (No. 20210276).

there are no inexpensive and convenient methods to measure 2-MeOE$_2$ levels. This study used hollow fiber liquid-phase microextraction (HF-LPME) and high-performance liquid chromatography-mass spectrometry (HPLC-MS) to improve sensitivity.

Materials and methods

Reagents and materials

The analytical reference 2-MeOE$_2$ was purchased from Sigma-Aldrich (Beijing, China). Ethinyl estradiol (IS) with a purity > 98% was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol (Fisher, Pittsburgh, PA, USA) was used for the HPLC analysis and urine sample preparation. Analytic-grade n-octyl alcohol (Tianjin Heng Xing Corporation, Hebei, China) was used to prepare urine extracts. A polyvinylidene difluoride (PVDF; Foshan, Guangzhou, China) hollow fiber membrane was used for HF-LPME. An Agilent 1200 liquid chromatography system (Agilent, Santa Clara, CA, USA) was used for all the analyses. An enzyme-linked immunosorbent assay (ELISA) kit for E$_2$ was purchased from Shanghai BlueGene Biotech Co., Ltd. (Shanghai, China).

Urine sample collection

This hospital-based case-control study of endometrial cancer was conducted at the Fourth Hospital of Hebei Medical University (Hebei, China). The study patients had newly diagnosed endometrial adenocarcinoma, which was confirmed by pathological examination postoperatively. Twenty-eight patients with endometrial cancer (45–74 years of age) were included in the study. Patients who received medical treatment 3 months before study enrollment and those with a history of cigarette smoking were excluded. Twenty-eight healthy women (46–65 years old) were included as controls. Healthy women underwent complete examinations, including ultrasonic examination of the liver, kidney, abdomen, and uterus at the Fourth Hospital of Hebei Medical University (Hebei, China). Serum tumor markers were negative in healthy women. All studies were conducted according to the protocols approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Hebei, China). Informed consent was obtained from all the patients and healthy controls.

Twenty-four-hour urine samples were collected in 1-L bottles containing 1 g of ascorbic acid to prevent oxidation. None of the women received exogenous estrogens. The urine volume was recorded immediately after collection. Aliquots of urines were stored at –20 °C until analysis.

Instruments and chromatographic and mass spectrometry conditions

All chromatographic analyses were performed using an Agilent 1200 liquid chromatography system. Chromatographic separation was performed on a Kromasil C18 column [150 mm × 4.6 mm (I.D.), particle size: 5 mm; Agilent]. The column temperature was maintained at 25 °C. Chromatographic separation was achieved isocratically using a mobile phase [water and methanol (8:92), v/v] supplemented with 0.1% acetic acid. The flow rate was set at 1 mL/min, and the injection volume was 10 μL. The total analysis time was 7 min for each run. Detection was performed using a 3200 QTRAPTM system (Applied Biosystems, Foster City, CA, USA) with a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and Turbo Ionspray interface. The instrument was operated using an electrospray ionization source in positive mode. Multiple reaction monitoring mode was used for quantification (Fig. 1). All instruments were controlled and synchronized using the Analyst

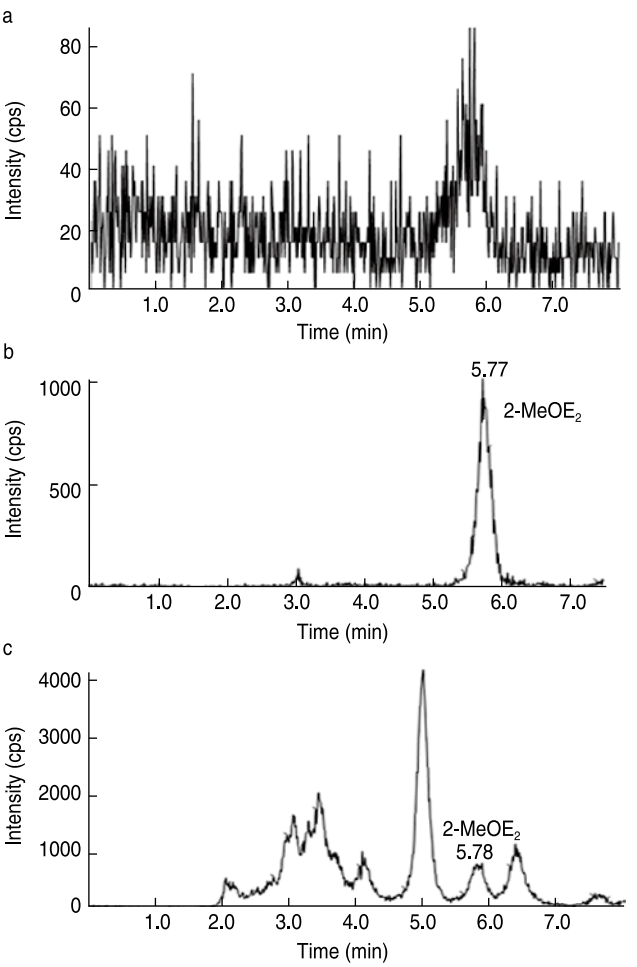

Fig. 1 Mass-spectrogram of 2-MeOE$_2$. (a) Control; (b) Artificial human urine; (c) Urine of patients with endometrial cancer

software (version 1.4.2; Applied Biosystems/MDS Sciex, Beijing, China).

Hydrolysis, extraction, and derivatization procedure

Because 2-MeOE$_2$ is mostly present in urine as glucuronide conjugate and small amounts of sulfate conjugate, a hydrolysis step was included. To a 140-mL aliquot of urine, 5.6 g of NaOH was added, followed by boiling for 10 min and centrifugation at 450 × g for 10 min. The precipitate was discarded, and concentrated hydrochloric acid was added to acidify the residual solution (pH 3.0). The residual solution was then diluted with water to a final volume of 140 mL.

The optimization procedure was conducted using 20 ng/mL of standard solutions. Extraction and preconcentration procedures were performed as previously described. At the end of the extraction, the hollow fiber extraction device was removed from the bottle, all sealed ends were carefully cut, and the organic acceptor phase solvent was carefully withdrawn into the microsyringe. Subsequently, 100 μL of methanol was slowly flushed through the lumen to simultaneously transfer analytes in the acceptor and membrane phases into a clean and dry polytef insert tube. The entire elution solution was evaporated to dryness at 90 °C under nitrogen gas.

Sodium bicarbonate buffer (100 μL, pH 9.0) and 100 μL of dansyl chloride solution (1 mg/mL in acetone) were added to the dried samples. After vortexing, the sample was heated at 60 °C for 5 min to form 2-MeOE$_2$ and dansyl chloride derivatives, respectively.

Ethinyl 2-MeOE$_2$ was dissolved in HPLC-grade methanol to produce an IS solution at a concentration of 143 pg/mL. To validate the method, three concentrations of the standard solution added to 2-MeOE$_2$ (10, 100, and 400 pg/mL) were used to prepare control urine samples.

Determination of E_2 using ELISA

The concentration of E_2 was measured using a microplate reader (VersaMax, Shanghai, China), according to the manufacturer's instructions. A standard curve was established to calculate E_2 concentrations in the samples.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). The results are expressed as medians. A comparison of the two groups was performed using a non-parametric test when the variance was uneven. Statistical significance was set at $P < 0.05$.

Results

Linearity, LOD, LOQ, and recovery

The linear range was 1.714–685.2 pg/mL, with a correlation coefficient of 0.996 using a weighted linear regression method. The calibration equation was as follows: A = 1.91 C + 2.91, where C is the concentration of 2-MeOE$_2$ (pg/mL). The SD of the slope and intercept were 0.0009 and 0.0004, respectively. The limits of quantification (LOQ) and limits of detection (LOD), defined as signal-to-noise ratios (S/N) of 10 and 3, respectively, were separately determined by five-fold replicate analysis. The LOD and LOQ were 0.14 pg/mL and 1.4 pg/mL, respectively.

Intra- and inter-day precision and stability

Table 1 presents the results of the accuracy and recovery of the proposed method. We measured the intra- and inter-day precision at various concentrations to assess the repeatability and reproducibility of the newly developed method. The relative standard deviations (RSD) of the intra-day precision of the three concentrations were 8.4%, 6.5%, and 7.2%, respectively (Table 1). Moreover, we evaluated the inter-day precision of the method by assessing sample concentrations at high, middle, and low linearity ranges over 6 consecutive days. The RSD values of the inter-day precision were 6.2%, 4.8%, and 5.6%, respectively (Table 1).

To evaluate freeze-thaw stability, samples were subjected to freezing for 24 h at –20 °C and thawed at room temperature (25–28 °C) for three cycles. The stability at freezing was assessed by storing the samples at –20 °C for 48 h, whereas the stability at room temperature (25–28 °C) was assessed by placing the samples at room temperature (25–28 °C) for 6 h. All RSD values for sample stability were < 7.8%.

Analysis of human urine samples (Table 2)

Concentrations of E_2 and 2-MeOE$_2$ in human urine

The distributions of E_2 and 2-MeOE$_2$ concentrations were abnormal. The median E_2 concentration in the case group was 3.38 ng/mL, whereas that in the control group was 2.34 ng/mL. The median 2-MeOE$_2$ levels in the case and control groups were 3.38 pg/mL and 9.85 pg/mL, respectively.

Table 1 Results of recovery rate and precision of the method (n = 6)

Concentration (pg/mL)	Relative recovery (%)	Inter-day RSD (%)	Intra-day RSD (%)
13.7	90.4	6.2	8.4
137.0	98.9	4.8	6.5
685.6	95.2	5.6	7.2

Table 2 Median concentration and quantity of E_2 and 2-$MeOE_2$ at 24 h in the urine of patients with endometrial cancer and healthy controls

Groups	Concentration		Content at 24 h		
	E_2 (ng/mL)	2-$MeOE_2$ (pg/mL)	E_2 (mg)	2-$MeOE_2$ (ng)	E_2/2-$MeOE_2$
Control (n = 28)	2.34	9.85	2.70	12.01	2.31
Case (n = 28)	3.38	3.38	4.40	6.77	9.91
P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Note: Performed by a non-parametric test

Levels of E_2 and 2-$MeOE_2$ at 24 h

The distributions of E_2 and 2-$MeOE_2$ levels at 24 h were abnormal. The median E_2 levels at 24 h in the case and control groups were 4.40 mg and 2.70 mg, respectively. The median 2-$MeOE_2$ levels in the case and control groups were 6.77 ng and 12.01 ng, respectively.

Comparison of E_2/2-$MeOE_2$ in patients and controls

2-$MeOE_2$ is a product of E_2 hydroxylation and methylation. The E_2/2-$MeOE_2$ ratio at 24 h was calculated. The E_2/2-$MeOE_2$ ratio at 24 h was abnormal. The E_2/2-$MeOE_2$ ratio was significantly higher in the case group (9.91) than that in the control group (2.31; $P < 0.05$).

Discussion

Urinalysis is widely used clinically because it is a simple procedure that provides critical information about disease processes and facilitates disease diagnosis, treatment, and prognosis. Although the urinary levels of 25 androgens and corticoids and 16 estrogens have been quantitatively determined using gas chromatography-mass spectrometry-selected ion monitoring [15], the measurement of 2-$MeOE_2$ concentration remains unresolved. We used HF-LPME and HPLC-MS to successfully measure 2-$MeOE_2$ levels. This method is simple, effective, and sufficient to determine 2-$MeOE_2$ levels in humans.

The exact endogenous 2-$MeOE_2$ concentrations in tissues are unknown, and the serum concentrations of 2-$MeOE_2$ are frequently reported in combination with 2-methoxyestrone. The reported plasma concentrations of 2-$MeOE_2$ in men, non-pregnant women, and pregnant women are 10–35 pg/mL, 18–138 pg/mL, and 216–10 690 pg/mL, respectively [16–17]. Recently, an analytical method developed to detect different methoxyestrogens reported the total concentration of 2-$MeOE_2$ in the serum to be (10.6 ± 7.91) pg/mL, and (2.5 ± 0.57) pg/mL in the luteal and follicular phases in pre- and postmenopausal women, respectively [18]. The concentration of the unconjugated form of 2-$MeOE_2$ was approximately half of the above values. In this study, the concentration of 2-$MeOE_2$ was measured in urine. The median 2-$MeOE_2$ level was 9.08 pg/mL in the control group, which is similar to the serum level reported previously.

2-$MeOE_2$ is endogenously formed from estradiol and has been reported to be a potent antiangiogenic and antitumor agent [19]. Regarding *in vitro* antiproliferative properties, the majority of 60 cell lines from human tumor cell lines are sensitive to 2-$MeOE_2$ with inhibitory concentrations between 0.08 and 5.0 μM [20]. The median 2-$MeOE_2$ concentration in the case group was 6.77 pg/mL, which was significantly lower than that of the control group. To exclude the influence of urine volume, 2-$MeOE_2$ levels at 24 h were compared. Thus, the reduction in 2-$MeOE_2$ was associated with a reduction in anticancer activity, which can be explained by the change in COMT.

Many tissues can actively produce 2-$MeOE_2$ because COMT is a ubiquitous enzyme found in different organs, such as the liver, kidney, intestine, stomach, spleen, brain, pancreas, and lungs [21]. Both COMT protein expression and activity are reduced in endometrial cancer tissues [22]. It is reasonable to assume that a decrease in 2-$MeOE_2$ levels is a risk factor for endometrial cancer.

The level of E_2 was measured using ELISA. E_2 is also associated with the occurrence of endometrial cancer [23]. Thus, the relative quantity of E_2 in the case group was significantly higher than that in the control group ($P < 0.05$). The E_2/2-$MeOE_2$ ratio in the case group (9.91) was significantly higher than that in the control group (2.31; $P < 0.05$). E_2 can induce endometrial cancer, whereas 2-$MeOE_2$ acts against endometrial cancer. Thus, the E_2/2-$MeOE_2$ ratio could be used as an indicator to identify patients with endometrial cancer in a high-risk population.

Acknowledgments

We would like to thank the Department of Gynecology and Institute of Hebei Cancer Research of the 4th Hospital of Hebei Medical University, China. We are particularly grateful to Ye Jiang for his excellent technical assistance. We are also grateful to the patients and women without cancer who participated in this study.

Funding

This work was supported by the Hebei Province Medical Science Research Key Project (No. 20210276).

Conflicts of interest

The authors indicated no potential conflicts of interest.

Author contributions

All authors contributed to data acquisition and interpretation and reviewed and approved the final version of this manuscript.

Data availability statement

Not applicable.

Ethical approval

Not applicable.

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DOI 10.1007/s10330-021-0510-0
Cite this article as: Zhao HH, Li JY, Liu Y, et al. Association of 2-methoxyestradiol levels with the occurrence and development of endometrial cancer in humans. Oncol Transl Med. 2022;8(4):191–195.