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Water-extracted tubers of *Cyperus rotundus* L. enhance endometrial receptivity through leukemia inhibitory factor-mediated expression of integrin αVβ3 and αVβ5

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ABSTRACT

Ethnopharmacological relevance: *Cyperus rotundus* L. (CR) has been traditionally used as an herbal medicine in Asian countries to treat diverse gynecological disorders. However, the potential therapeutic effect of CR on endometrial receptivity for successful embryo implantation to treat female infertility has not been fully studied.
Aim of study: The aim of this study was to evaluate the effect of water-extracted CR on endometrial receptivity by investigating the expression of leukemia inhibitory factor (LIF) and integrins, cell adhesion, and embryo implantation using mifepristone (RU486; RU)-induced implantation failure model.
Materials and methods: The water extract of CR was prepared and fingerprinted using high-performance liquid chromatography (HPLC). For the expression and regulation of LIF, reverse transcription polymerase chain reaction (RT-PCR) and western blotting were performed in CR-stimulated Ishikawa cells. To evaluate LIF-mediated integrin expression, knockdown of LIF by shRNA was performed in Ishikawa cells. The effect of CR on endometrial receptivity was determined by an *in vitro* adhesion assay between JAr cells and CR-induced Ishikawa cells. *In vivo*, C57BL/6 female mice (n = 7 per group) orally received CR (31.68 mg/kg/day), a similar dose as used clinically. Seven days after CR treatment, all female mice were caged with male mice until pregnancy was verified. On day 4 of pregnancy, RU (4 mg/kg) was injected subcutaneously to induce embryo implantation failure.
Result: CR increased the expression of LIF through the phosphatidylinositol-3-kinase/ protein kinase B (PI-3K/AKT) signaling pathway in Ishikawa cells. In addition, CR enhanced adhesion of JAr cells onto Ishikawa cells by inducing the expression of LIF-dependent integrins αVβ3 and αVβ5. Furthermore, CR improved the number of implantation sites in pregnant mice despite RU injection.
Conclusion: CR increased the expression of LIF-mediated integrins αVβ3 and αVβ5 on the surface of endometrial cells, which is associated with adhesion of trophoblastic cells to endometrial cells for blastocyst implantation. Our findings provide evidence that CR has therapeutic potential against poor endometrial receptivity.

1. Introduction

Cyperus rotundus L. (CR) is a perennial plant belonging to the Cyperaceae family, which is widely distributed in Africa, southern and central Europe, and southern and eastern Asia. The roots and tubers of CR have been used as an herbal medicine in China, Japan, India, and Korea for treating diverse gynecological disorders, such as amenorrhea, irregular menstruation, and pelvic pain (Chen et al., 2014a, b; Pirzada et al., 2015). The decoction of CR was used for treating female infertility in Asian countries, including Vietnam (Cambie and Brewis, 1997). In addition, a clinical research report indicated that CR is one of the most popular herbal remedies prescribed by traditional Chinese doctors for treating female infertility in Taiwan (Hung et al., 2016). Although restoration of a regular menstrual cycle is regarded as a

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mechanism underlying the anti-infertility action of CR (Cambie and Brewis, 1997; Pirzada et al., 2015), there is no direct experimental evidence supporting the ameliorative effect of the herb on female infertility.

Female infertility can be caused by failures at various steps, including ovulation, fertilization, embryo development, embryo transport, and implantation (Jose-Miller et al., 2007). Recent development of assisted reproductive technology (ART) has overcome the majority of infertility causes; however, impaired implantation remains an unmet need (Revel, 2012; Wang and Dey, 2006). For successful implantation, a receptive endometrium, a functionally developed embryo, and their synchronized interaction are required (Koot and Macklon, 2013). Although there have been significant improvements in understanding endometrial receptivity, molecular mechanisms regulating endometrial-blastocyst interaction remain poorly understood (Aplin, 2006). In addition, the therapeutic options for treatment of implantation failure are limited (Revel, 2012). From this point of view, we have strived to find novel candidates from traditional medicinal herbs for improving endometrial receptivity (Choi et al., 2016; Kim et al., 2016).

In this study, we report for the first time that the water-extracted tubers of CR stimulate endometrial receptivity in *in vitro* and *in vivo* experiments. The results of this study also demonstrate that leukemia inhibitory factor (LIF)-mediated expression of integrins αVβ3 and αVβ5 is crucial for the effects of CR on endometrial receptivity. Therefore, we conclude that the enhanced receptivity of the endometrium may explain the effects of CR on female infertility.

2. Materials and methods

2.1. Materials

The progesterone receptor antagonist, mifepristone (RU486; RU), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors of specific signaling pathways, including LY294002 (for phosphatidylinositol-3-kinase; PI-3K), U0126 (for mitogen-extracellular signal-regulated kinase/extracellular signal-regulated kinase; MEK/ERK), SB203580 (for p38 mitogen-activated protein kinases; p38), and SP600125 (for c-Jun *N*-terminal kinase; JNK), were obtained from Merck Millipore (Billerica, MA, USA).

2.2. Plant material and extract preparation

The tubers of CR were purchased from Omniherb Co. (Daegu, Korea) and authenticated by a botanical expert working at the company. The voucher specimen (no. DC-H27) was deposited at the Healthy Aging Korean Medicine Research Center, Pusan National University (Yangsan, Korea). The extraction method is shown in Fig. 1A. Briefly, the tubers of CR (100 g) were decocted with distilled water (1 L) for 2 h at 100 °C. The decoction was extracted with 70% ethanol at 4 °C overnight and centrifuged at 4000 rpm for 15 min to remove polysaccharide. Then, the supernatant was concentrated with an Eyela rotary evaporator (Tokyo Rikakikai Co., Tokyo, Japan) and lyophilized with a freeze-drier (Labconco, Kansas City, MO, USA) to give a powder (11.8821 g). The powder was freshly dissolved in distilled water before using in experiments.

2.3. Fingerprinting high-performance liquid chromatography (HPLC) analysis

The phytochemical property of CR was examined by HPLC analysis, comparing it to known compounds that included nootkatone and α-cyperone, according to previous protocols with some modifications (Pirzada et al., 2015; Priya Rani and Padmakumari, 2012). The HPLC experiment and data analysis were conducted with an Agilent 1200 series system (Agilent Technologies, CA, USA) and LC solution software (version 1.24), respectively. The analytical column was an AkzoNobel KR10-5C18 column (4.6 × 250 mm), which was maintained at 30 °C. The mobile phases were solvent A (0.1% Formic acid in Water) and solvent B (Methanol). The gradient flow was as follows: (A)/(B) = 50-20/50–80 (0–30 min) → (A)/(B) = 20-10/80–90 (30–40 min) → (A)/(B) = 10-0/90–100 (40–42 min) → (A)/(B) = 0-0/100-100 (42–50 min) → (A)/(B) = 0–50/100-50 (50–65 min) → (A)/(B) = 50-50/50-50 (65–70 min). The analysis was carried out at a flow rate of 1 mL/min with UV detection at 254 nm. The column injection volume was 10 μL. Standard compounds (*i.e.,* nootkatone and α-cyperone) were dissolved in distilled water (10 μM). The standard solutions and samples were filtered with a 0.45 μm membrane filter before subjecting them to HPLC analysis.

2.4. Cell culture

The endometrial Ishikawa cell line was kindly provided by Dr. Jacques Simard (CHUL Research center, Québec, Canada). The cells were maintained at 37 °C in a 5% CO_2 / 95% air atmosphere in Dulbecco's Modified Eagle Medium (DMEM; Welgene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin (PSA, Thermo Fisher Scientific). The choriocarcinoma JAr cells, obtained from the Korean Cell line Bank (Seoul, Korea), were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene) containing 10% FBS and 1% PSA.

2.5. Cell viability assay

The cytotoxicity of CR was examined using a 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, MO,

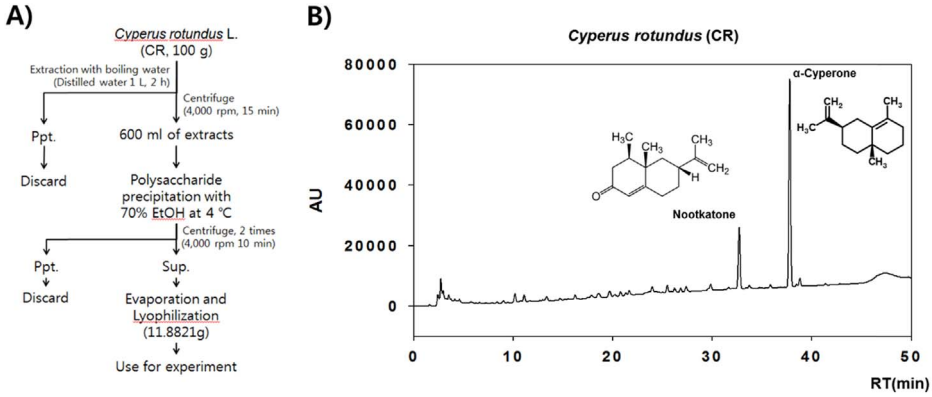

Fig. 1. Method for water-extraction and fingerprinting of *C. rotundus* (CR). **(A)** The schematic of the water-extraction procedure for CR. **(B)** The water-extract of CR was analyzed by HPLC.